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<p>(54) Title: <b>METHODS AND AGENTS FOR MODULATING THE IMMUNE RESPONSE AND INFLAMMATION INVOLVING MONOCYTE AND DENDRITIC CELL MEMBRANE PROTEINS</b>          (57) Abstract  <p>Methods and agents are provided to decrease or increase the migration of dendritic cells for the suppression or enhancement, respectively, of the development of immunity and the immune response, by modulating the dendritic cell membrane proteins p-glycoprotein (MDR-1) and tissue factor. Agents which suppress migration have utility in the treatment of immunologically-mediated and inflammatory diseases such as graft rejection, contact dermatitis, seasonal allergies, asthma, and food allergies. Agents which enhance migration are useful for increasing the effectiveness of vaccines. Agents are also disclosed which enhance the migration of monocytes, useful in the treatment of chronic inflammatory diseases. Methods are also provided for identifying useful agents by measuring the effect on dendritic cell migration of agents which modulate p-glycoprotein and tissue factor activity, as well as the effect of agents on monocyte migration.</p> </p>		

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**METHODS AND AGENTS FOR MODULATING THE IMMUNE RESPONSE AND  
INFLAMMATION INVOLVING MONOCYTE AND DENDRITIC CELL  
MEMBRANE PROTEINS**

**RESEARCH SUPPORT**

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**FIELD OF THE INVENTION**

- 10    The present invention relates generally to methods and agents for enhancing or suppressing the development of immunity and the immune response by modulating the migration of dendritic cells, and methods for identifying agents with migration modulating activity. Methods and agents for the enhancement of monocyte migration out of chronic inflammatory foci are also provided.

15                   **BACKGROUND OF THE INVENTION**

- The development of immunity to a specific antigen and the subsequent immune response on re-encountering that antigen involve multi-step interactions between several immune cell types and co-stimulatory molecules with critical spatial and temporal components. In brief, an antigen encountered by antigen-presenting cells is taken up, processed, and its peptides  
20     subsequently prime T lymphocytes to generate antigen-specific T lymphocytes (e.g.,

cytotoxic and memory cells), and through the interaction with B cells, result in the formation of antigen-specific antibodies. Immune responses are initiated after antigen-presenting cells, particularly dendritic cells (DC), capture antigens in organs such as skin, lung and gut, and migrate via afferent lymphatic vessels to draining lymph nodes. T lymphocytes continually recirculate through lymph nodes, so the newly arrived, antigen-bearing DC become positioned to select lymphocytes that bear receptors for the presented antigens (1). For example, when skin is transplanted (2), or a contact allergen such as poison ivy is applied (3), epidermal DC (also termed Langerhans cells) migrate from their epithelial locations and carry antigens or allergens to the lymph node. If the lymphatic conduits to the draining lymph nodes are severed, the immune system does not become primed to the antigen (2). As they migrate, the DC undergo maturation, increasing their expression of molecules involved in antigen presentation, including major histocompatibility complex (MHC) II products, CD80 (B7-1), and CD86 (B7-2) (4, 5).

Thus, dendritic cell migration is a critical step in both the induction of immunity and in the immune response. Dendritic cell migration is essential for the successful induction of an immune response to an administered vaccine, for eliciting antibody production and cell-mediated immunity against infectious microorganisms and arising neoplastic cells within the body. Likewise, dendritic cell migration is responsible for diseases involving an exaggerated, inappropriate or undesired immune response such as allergic asthma, food allergies, contact dermatitis, seasonal allergies, and autoimmune diseases such as psoriasis, in which known or as-yet undescribed antigens are transported to T lymphocytes.

White blood cells known as monocytes are precursors of dendritic cells and tissue macrophages. These cells migrate throughout the tissues, and may develop into dendritic cells, as described above, or macrophages, which remain in the tissues. Excessive retention and pro-inflammatory activity of macrophages in certain tissues is implicated in acute and chronic inflammatory diseases, including atherosclerosis, rheumatoid arthritis, and granulomatous diseases.

Blockage of or interference with dendritic cell migration interferes with the immune response. Blockage has been achieved by the use of antibodies against certain dendritic cell proteins. Before leaving the epidermis, dendritic cells down-regulate E-cadherin, allowing retraction from neighboring keratinocytes (31). In the presence of anti- $\alpha_6$  integrin antibody, epidermal DC up-regulate MHC II and retract from neighboring keratinocytes, and fail to migrate out of the epidermis (32). Another adhesion molecule, CD44, is also used by DC during mobilization from the epidermis (33).

It is towards improved means for specifically modulating the migration of dendritic cells from the periphery to lymphatic vessels for both the positive and negative control of the immune response, and blockage of monocyte migration to control the inflammatory response, that the present invention is directed.

The citation of any reference herein should not be deemed as an admission that such reference is available as prior art to the instant invention.

## SUMMARY OF THE INVENTION

The inventors herein have made the surprising and unanticipated finding that certain dendritic cell membrane proteins, namely p-glycoprotein, also known as MDR-1, the transporter responsible for multi-drug resistance, and tissue factor (TF), a procoagulant protein, are involved in the migration of dendritic cells (DCs) from the peripheral tissues to the lymphatic vessels. Alteration of dendritic cell migration activity by manipulating these surface proteins is useful for both enhancing and suppressing the immune response. Furthermore, the migration of monocyte-derived cells out of tissues has been found to be inhibited by agents which interfere with monocyte p-glycoprotein.

Thus, in accordance with the present invention, methods for the modulation of immunity and the immune response in a mammal is provided by an agent which is capable of interacting with the dendritic cell membrane proteins p-glycoprotein and tissue factor, and altering the migration of dendritic cells from the peripheral tissues to the lymphatic vessels. In one embodiment, modulation results in decreasing the migration of dendritic cells and hence the suppression of immunity or of an immune response. Agents useful for the practice of this aspect of the invention include antibodies and antibody fragments which bind to p-glycoprotein, agents which block transport activity of p-glycoprotein, antisense oligonucleotides to p-glycoprotein and antagonists of p-glycoprotein. Preferred agents are those which reverse multi-drug resistance, such as calcium channel blockers, reserpine, certain antimalarial drugs, cyclosporins, phenothiazines, tamoxifen and its metabolites, certain antibiotics, antisense oligonucleotides, and other compounds known to antagonize the activity of p-glycoprotein. Useful antibodies include MRK-16, UIC2, 4E3, and their fragments.

In a further embodiment, antagonists of tissue factor are useful for the suppression of the development of immunity and of the immune response. Such antagonists include antibodies and antibody fragments to tissue factor, tissue factor antisense oligonucleotides, fragments of tissue factor, and compounds known to antagonize tissue factor such as dilazep and retinoic acid.

Suppression of the immune response is useful for the treatment of several diseases and conditions. For example, allergic reactions, graft-vs-host disease, transplant rejection, autoimmune diseases, progression of HIV infection, contact dermatitis, and food allergy are treatable by the methods and agents of the present invention. The agents may be administered by appropriate routes including topical, parenteral, ophthalmic, nasal, pulmonary, etc., for delivery to the site of encounter of the dendritic cells with the antigen and to prevent their subsequent migration to the lymphatic vasculature.

In a further embodiment, certain methods and agents of the present invention may be used which enhance the migration of dendritic cells from the peripheral tissues to the lymphatic vessels. These methods and agents promote the development of immunity or of an immune response to a newly encountered antigen and enhance immunity or the immune response on re-encountering an antigen. Agents useful in the practice of this aspect of the present invention are agonists of the dendritic cell membrane proteins p-glycoprotein and tissue factor. Examples of useful agents include bromocriptine, N-acetyl-leucine-leucine-tyrosine amide, and cytarabine for p-glycoprotein. In a preferred embodiment, the agent is co-administered with an immunogen to enhance the development of an immune response to said immunogen, for example, when the immunogen is a vaccine.

In a further aspect of the present invention, methods for the identification of agents useful for the modulation of dendritic cell migration is provided. These methods comprise the steps of first identifying an agent capable of interacting with the dendritic cell membrane proteins p-glycoprotein and tissue factor, followed by determining the extent of modulation of the migration of dendritic cells in vitro or in vivo by the agent. These methods identify agents useful for modulating the development of immunity or an immune response.

In another aspect of the present invention, methods for the modulation of inflammation in a mammal are provided by an agent which is capable of enhancing the function of the monocyte membrane protein p-glycoprotein, and increasing the migration of inflammatory monocytic cells from the tissues. Agents useful for the practice of this aspect of the invention include the aforementioned agonists of p-glycoprotein. Increasing monocyte migration out of tissues is useful in the treatment of such inflammatory conditions as atherosclerosis, rheumatoid arthritis and granulomatous diseases.

In a still further aspect of the present invention, methods for the identification of agents useful for the augmentation of monocyte migration out of tissues is provided. These methods comprise the steps of first identifying an agent capable of enhancing the monocyte membrane protein p-glycoprotein, followed by determining the extent of augmentation of the migration of monocytes in vitro or in vivo by the agent. These methods identify agents useful for treating inflammatory conditions and diseases.

These and other aspects of the present invention will be better appreciated by reference to the following figures and Detailed Description.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1 A-B.** Effect of anti-MDR-1 monoclonal antibodies (mAbs) on transendothelial migration of mononuclear phagocytes. (A) Monocytes were allowed to migrate across endothelial monolayers with or without addition of anti-MDR-1 mAb MRK16, or mAb to  $\beta_2$  integrin (IB4) or CD31 (hec 7). Cultures were incubated for 1.5 hr. (B) To examine subsequent basal-to-apical transmigration, monocytes were first allowed to accumulate beneath the endothelium in the absence of added mAb for 1.5 hours, followed by addition of mAbs (all IgG2a; MRK16 used as  $F(ab')_2$  fragments) at the indicated concentrations; in some samples, monocytes were preincubated with MRK16 or isotype-matched control mAb hec 1 against cadherin 5 (Pre-MRK16 and Pre-Hec 1) before addition to the endothelium. After 48 hr, cultures were fixed for analysis. Data are plotted relative to control levels of reverse transmigration (no mAb added), in which 50% of the subendothelial MP retrace the endothelium in 48 hr. Bars represent means  $\pm$  SD from three to ten experiments, except UIC2 which was tested in a single experiment.

**Figure 2.** Effect of anti-MDR-1 mAb on emigration of DC and T lymphocytes from skin explants. Explants of human skin were floated in culture medium without added mAb ( $n = 10$ ) or in medium containing anti-MDR-1 mAbs MRK16 ( $n = 10$ ) or UIC2 ( $n = 3$ ), anti-cadherin 5 mAb hec 1 ( $n = 5$ ), anti-CD31 mAb hec 7 ( $n = 3$ ), anti-MHC I mAb W6/32 ( $n = 3$ ), anti-CD18 mAb IB4 ( $n = 2$ ), verapamil ( $n = 3$ ), or the vehicle control for verapamil, 0.03% methanol (Vehicle,  $n = 1$ ). After three days of incubation, DC and T lymphocytes that appeared in the culture medium were collected and counted as previously described (7). Each experiment was conducted in triplicate;  $n =$  number of experiments in which condition was

included. The number of emigrated cells recovered from individual control explants was typically  $5 \times 10^5$ . To compare data from different experiments, the mean number of emigrated cells in the absence of added mAb in each experiment was set equal to 1.0 and relative values were obtained for the remaining data.

- 5 **Figure 3 A-C.** Expression of MDR-1 in situ by DC. (A) Epidermis derived from explants cultured with anti-MDR-1 mAb MRK16 were fixed and Cy3-conjugated anti-mouse IgG was added to detect expression of MDR-1 (red). Addition of Cy3-labeled detection antibody to skin incubated in the presence of nonbinding control mAb (Hec1) showed no staining (not shown). (B) Immunostaining of same sample using FITC-conjugated anti-MHC II mAb  
10 (green) identified dendritic cells. (C) Doubly-exposed frame to examine co-localization of glycoprotein and MHC II on dendritic cells (yellow). Bar is 10  $\mu$ m.

- Figure 4 A-E.** Expression of functional MDR-1 by emigrated DC. (A-C) MDR-1 expression in emigrated skin cells was examined by flow cytometry using double-staining with anti-MDR-1 mAb and the DC lineage marker MHC II (A, B) or the T lymphocyte lineage  
15 marker CD3 (C). Quadrants were marked based on the level of fluorescence intensity observed in samples stained with negative control mAbs. (D) For studies measuring efflux, emigrated cells were loaded with the dye 3,3'-diethyloxycarbocyanine iodide (DiOC<sub>2</sub>) (- - -, no dye added) and cultured at 37 °C for 90 minutes without addition of mAb (filled profile), in the presence of UIC2 mAb (bold line), or kept at 4 °C for this duration (thin line).  
20 Analysis of efflux in the DC fraction of emigrants was assessed by double labeling with PE-conjugated anti-MHC II mAb for flow cytometry. Figure shown was gated on MHC II<sup>+</sup> cells. (E) Membranes prepared from purified, emigrated DC, PBMC, or HUVEC were

immunoblotted with anti-MDR-1 mAb C219. Numbers to the left of bands are molecular weight markers.

**Figure 5 A-F.** Effect of verapamil on the efflux of the p-glycoprotein synthetic substrate 3,3'-diethyloxacarbocyanine iodide ( $\text{DiOC}_2$ ) from dendritic cells. Panels A and B show the fluorescence intensity of dendritic cells before loading with  $\text{DiOC}_2$ . In these cells, the FL1 staining intensity (x-axis) is low. Panels C-F show dendritic cells incubated under various conditions after loading with  $\text{DiOC}_2$ , which fluoresces on the FL1 channel. Panels B-F show cells after labeling for the cell surface marker MHC II (FL2 channel). Cell that shift  $10^3$  staining intensity on the FL2 channel are dendritic cells. For efflux measurements, dendritic cells emigrated from skin were loaded with the dye and analyzed immediately for dye content (time 0, Panel C), or cultured for 90 minutes at  $37^\circ\text{C}$  without additives (D) or in the presence of verapamil ( $10\text{ }\mu\text{g/ml}$ ) (Panel E) or the anti-p-glycoprotein mAb UIC2 ( $10\text{ }\mu\text{g/ml}$ ) (Panel F).

**Figure 6 A-D.** Retention of DC in the epidermis after treatment with MDR-1 antagonists.

(A) Epidermal sheets were stained with anti-MHC II mAb to enumerate DC before the onset of culture or after three days of culture in the absence of mAb (no mAb,  $n = 7$ ), or in the presence of anti-cadherin 5 mAb hec 1 ( $n = 3$ ); anti-MHC I mAb W6/32 ( $n = 3$ ); anti-CD31 mAb hec 7 ( $n = 3$ ); anti-MDR-1 mAb MRK16 ( $n = 6$ ); anti-MDR-1 mAb UIC2 ( $n = 1$ ); verapamil ( $n = 2$ ); or the vehicle control for verapamil, 0.03% methanol (Vehicle,  $n = 1$ ).  $n =$  number of experiments in which each condition was examined. DC were counted from *en face* examinations of epidermal sheets in 16 to 20 high-power fields per experiment. Percent reduction in DC density was calculated by comparing the number of DC in cultured explants

to the mean number present in a portion of the same skin sample before culture (typically 75 cells/field). **B-D)** Photomicrographs show the distribution of DC within the epidermis before culturing of explants (**B**), after three days of culture under control conditions (no mAb) (**C**), and after three days of culture in the presence of MRK16 (**D**). Bar is 20  $\mu$ m.

- 5 **Figure 7.** Effect of anti-MDR-1 mAb on the maturation of DC. The levels of MHC II expressed by epidermal DC were analyzed in gated epidermal suspensions by flow cytometry before the onset of culture (Day 0) or after three days of culture in the absence of mAb or in the presence of MRK16. Single cell suspensions of the epidermis were prepared by digestion with dispase followed by trypsin. Keratinocytes and other skin cells were excluded from the  
10 analysis by setting a gate to include only MHC II<sup>+</sup> cells.

- Figure 8.** Effect of anti-tissue factor monoclonal antibody (mAb) on emigration of dendritic cells (DC) and T lymphocytes from skin explants. Explants of human skin were floated in culture medium without added mAb or in medium containing anti-tissue factor mAb VIC7 or control mAbs against cadherin 5 or carcinoembryonic antigen (CEA). After three days of  
15 incubation, DC and T lymphocytes that appeared in the culture medium were collected. Cumulative results from three independent experiments are shown. Each experiment was conducted with 3-4 replicates per parameter tested. The number of emigrated cells recovered from individual control explants were typically  $5 \times 10^5$ . To compare data from different experiments, the mean number of emigrated cells in the absence of added mAb in each  
20 experiment was set equal to 1.0 and relative values were obtained for the remaining data.

**Figure 9 A-B.** Analysis of expression of p-glycoprotein-related transporters in mice. A) Dendritic cells emigrated from wild-type or MRP knock-out mouse ear explants were loaded with the fluorescent transporter substrate Fluo-3 and cultured at 37° C for 60 minutes (open profile) or kept at 4° C for this duration (filled profile). Analysis of efflux in the DC fraction of emigrants was assessed by double labeling with PE-conjugated anti-MHC II mAb for flow cytometry. Figure shown was gated on MHC II<sup>+</sup> cells. B) MRP expression in emigrated skin cells was examined by flow cytometry in permeabilized cells using double-staining with anti-MRP mAb MRPr1 (Signet Laboratories) or rat control mAb R35-95 (Pharmingen), followed by FITC-conjugated anti-rat mAb for detection.

**Figure 10 A-B.** Effect of MRP antagonist MK 571 on emigration of DC from skin explants. Explants of human skin or explants of the dorsal aspect of mouse ear were floated in culture medium with or without addition of MK 571 (25 µM). Murine emigrated dendritic cells were collected and explants were transferred to fresh medium daily (with or without MK 571) and were analyzed over 5 days. Human skin explants were analyzed on day 2, with a medium change or fresh addition of drug at day 1. Each experiment was conducted using 3 (human) to 10 (mouse) replicates and was performed on at least two independent occasions.

**Figure 11 A-B.** Contact sensitization in MRP knock-out mice or after administration of MRP antagonist to wild-type mice. A) Twenty five microliters of FITC dissolved in acetone:dibutylphthalate (8 mg/ml stock) was applied to the dorsothoracic area of shaved mice. Twenty four hours later, draining lymph nodes were collected, disaggregated to

obtain single cell suspensions, and stained for the dendritic cell marker CD11c. CD11c was detected using biotinylated anti-CD11c and streptavidin-conjugated R-phycoerythrin. Data are from a single experiment performed in quadruplicate. B). Mice were injected with 1 mg MK 571 intraperitoneally before application of FITC and analysis as described in (A). Data are from 2 experiments, each performed with triplicate samples.

### DETAILED DESCRIPTION OF THE INVENTION

The following terms shall have the definitions set out herein. The terms “immunity” or “development of immunity” are used generally to refer to the initial encounter of the immune system with an antigen and the development of a specific immune response to the antigen.

10 This entails, in brief, the uptake of the antigen by antigen-presenting cells (APCs), especially dendritic cells (DC), processing of the antigen, and presentation of antigen peptides in a complex with MHC molecules, to T lymphocytes, with the subsequent formation of antigen-specific T lymphocytes, including memory, helper, and cytotoxic T lymphocytes, as well as antibody-producing B lymphocytes. The term immune response is generally used herein to

15 refer to the response of the immune system to a subsequent encounter with an antigen to which immunity has been previously established. One aspect of this encounter is the uptake of the antigen by APCs, migration to draining lymphoid organs including lymph nodes and the spleen, and subsequent presentation to memory T lymphocytes that circulate in lymphoid tissues.

20 The terms “inflammation” and “inflammatory response” refer to the acute or chronic response to a known or unknown antigen in which a particular general or local site within the body exhibits adverse sequelae of the accumulation of white blood cells, including monocytes, and

their secreted mediators, producing a range of effects of varied intensities including pain, tenderness, edema, destruction of tissue, organ failure, asthma, and urticaria (hives). In the case of atherosclerosis, a chronic inflammatory disorder of the arterial wall, accumulation and retention of monocytes in the intimal tissue of the artery leads to lipid-laden plaque formation and danger of thrombosis.

As described above, the present invention relates to methods and agents for the modulation of the immune response involving the dendritic cell membrane proteins p-glycoprotein and tissue factor. As will be seen in the examples below, these proteins have been discovered to have an important physiological role in the migration of dendritic cells from the peripheral tissues, a location where antigen is encountered, to the lymphatic vessels, where interaction of the dendritic cell with T lymphocytes induces immunity or an immune response. Manipulation of these proteins to antagonize or agonize their activities has been found to be useful for decreasing or increasing, respectively, the ability of the dendritic cell to migrate to the lymphatic vessels and thus modulate the development of immunity or an immune response to an antigen or antigens encountered by dendritic cells.

p-Glycoprotein, also known as MDR-1, is a large plasma membrane protein that functions as an ATP-driven chemotherapeutic drug efflux pump. The endogenous substrate(s) for the p-glycoprotein transporter has not yet been identified, but based on structure-activity relationships of numerous compounds, are believed to include cationic, hydrophobic molecules with at least two planar rings and a molecular weight of 400-1500 (40). Endogenous substrates also likely include modified phospholipids, such as phospholipids that are involved in cell signaling. The presence of p-glycoprotein on cells confers multi-drug

resistance ("MDR"); in cancer cells, p-glycoprotein pumps out of the cell chemotherapeutic agents and thus renders cancer cells resistant to chemotherapy (36). Thus, to enable or improve cancer chemotherapy in resistant tumors, p-glycoprotein has been a target for pharmacologic intervention to disable the pump (36). Numerous compounds have been found which antagonize the activity of the pump, and several are in use or in clinical development as adjunct therapy with cancer chemotherapeutic agents to block resistance. Prophylactic use has also been suggested to prevent the appearance of a drug resistant phenotype.

Several gene products that are highly homologous to p-glycoprotein also confer drug resistance and can transport a similar profile of substrates as p-glycoprotein. Examples of these related proteins include cMOAT (canalicular multiple organic anion transporter), LRP (lung resistance protein), and MRP (multi-drug resistance related protein). Expression of these p-glycoprotein-related gene products in monocytes or dendritic cells of humans or other mammalian species may confer the same function as the MDR-1 gene product. Therefore, p-glycoprotein as defined herein embraces the MDR-1 gene product or the gene product(s) of structurally and functionally related transporters that mediate dendritic cell migration in humans and other mammals.

Methods and agents which have been found to effectively antagonize the activity of the p-glycoprotein protein are useful in the practice of the present invention for antagonizing p-glycoprotein and thus inhibiting dendritic cell migration and suppressing immunity or an immune response. These methods and agents comprise several approaches, including but not limited to antibodies and fragments which bind to p-glycoprotein, compounds which reverse

multi-drug resistance, and means to reduce the expression of p-glycoprotein. These methods and agents may be used singly or in combination. As will be described in more detail below, administration may be targeted to a particular location of the body where the activity is desired, for example on or in an cutaneous lesion such as psoriasis or contact dermatitis, and  
5 delivery by aerosol or powder to the bronchi or lungs in the case of asthma.

Suppression or down-regulation of immunity and the immune response has utility in the prophylaxis and treatment of numerous diseases and conditions. Methods and agents of the present invention are useful as prophylaxis against the exposure of an individual to a known immunogen or allergen against which an immune response would be highly undesirable. For  
10 example, acute exposure to a causative agent of contact dermatitis, such as might occur in an industrial setting, for example, exposure to protein-modifying metals and their salts such as nickel and chromate as a result of a chemical spill, or release of a gaseous or airborne particulate allergen; a further example is topical or pulmonary exposure to urushiol, from contact with poison ivy or inhalation of smoke from burning foliage. Immunity to accidental  
15 oral consumption of a potent immunogen may be blocked by the methods and agents of the present invention. An individual exposed under such conditions can prevent the delivery of the antigen to the lymph nodes by the methods and agents of the present invention.

Wider utility of the methods and agents of the present invention are found in the treatment of acute and chronic conditions mediated by the initial or repeat exposure of the immune system  
20 to a known or as-yet unidentified antigen causative of a condition or disease. A role for dendritic cells in the development of immunity has been established; the role of dendritic cells in the subsequent immune response has been established for many antigens and is

suspected in others. The agents of the present invention that suppress the migration of dendritic cells are useful for one or both of these aspects, and especially in the type of immune response known as delayed-type hypersensitivity and more particularly, contact hypersensitivity. Examples of known immunogens that cause disease include pollen and other airborne allergens which cause allergic rhinitis and other seasonal allergic reactions, hay fever, allergic conjunctivitis; arthropod-borne antigens such as dust mites and insect secretions such as those that cause the reaction to mosquito bites; foods that cause allergies, etc. In numerous autoimmune diseases, suspected antigens to which an immune response is responsible for the pathology have been identified; in others, the antigens are probable, and in others, they are uncertain. Examples of as-yet identified immunogens responsible for pathological inflammatory conditions include food allergies of unknown cause, irritable bowel syndrome, rheumatoid arthritis, psoriasis and a large number of autoimmune diseases which are believed to be initiated and maintained by endogenous antigens. In a further utility of the present invention, means to block an immune response to transplanted antigens, in the form of acute rejection, is provided. This may be used as an adjunct to immunosuppressive and other agents routinely used in transplantation.

The methods and agents of the present invention are not limited to, but are particularly useful in, the treatment of inflammatory conditions where the acute or chronic exposure to antigen occurs in the periphery, such as the skin, as well as the gut and the lungs.

As mentioned above, one particular condition amenable to treatment by the methods and agents of the present invention is in the area of transplantation. A critical role has been identified in the interaction of antigen-presenting cells, dendritic cells in particular, with T

lymphocytes in initiating rejection. It is well established that dendritic cells mediate allograft rejection, since experimental techniques used to deplete allografts of endogenous dendritic cells do not give rise to subsequent rejection responses in recipients. However, it is not clinically feasible to deplete allografts of endogenous dendritic cells. Because it is the migration of dendritic cells to the graft recipient's lymph nodes and spleen that ultimately results in graft rejection, clinically feasible methods to block dendritic cell migration to these lymphoid tissues would prevent graft rejection. Suppression of the migration of dendritic cells by agents which antagonize p-glycoprotein or tissue factor and thus inhibit their interaction with T lymphocytes blocks the development of the immune response and thus are useful for averting graft rejection.

Another disease particularly amenable to both prophylaxis treatment by the methods and agents of the present invention is HIV. Transepithelial infection by HIV is believed to be mediated by dendritic cells, which become infected with the virus and carry the virus to lymph nodes where interactions between dendritic cells and T lymphocytes as described above lead to infection of T lymphocytes. It is known that bone marrow-derived dendritic cells facilitate the creation of a population of CD 4+ T lymphocytes, which are a major reservoir of HIV infection. Methods and agents of the present invention which block the migration of dendritic cells from peripheral sites, where dendritic cell HIV infection occurs, can interrupt the endogenous spread of infection; suppressing further dendritic cell migration inhibits the dendritic cell-mediated fostering of infected T lymphocyte populations.

In one embodiment of the present invention, antibodies or antibody fragments to p-glycoprotein may be administered to inhibit dendritic cell migration. Antibodies may be

made which bind to the extracellular portion of the p-glycoprotein molecule. Non-limiting examples of suitable antibodies include monoclonal antibodies MRK16 (24), 4E3 (23), and UIC2 (22). Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. Other fragments of such

5 antibodies capable of binding to an epitope on the p-glycoprotein molecule are embraced herein. The anti-p-glycoprotein antibodies of the invention may be cross reactive, *e.g.*, they may recognize p-glycoprotein from different species. Polyclonal antibodies have greater likelihood of cross reactivity. Alternatively, an antibody of the invention may be specific for a single form of p-glycoprotein, such as murine p-glycoprotein. Preferably, such an antibody

10 is specific for human p-glycoprotein.

Various procedures known in the art may be used for the production of polyclonal antibodies to p-glycoprotein. For preparation of monoclonal antibodies directed toward p-glycoprotein, or fragment thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the

15 hybridoma technique originally developed by Kohler and Milstein [*Nature* **256**:495-497 (1975)], as well as the trioma technique, the human B-cell hybridoma technique [Kozbor et al., *Immunology Today* **4**:72 1983]; Cote et al., *Proc. Natl. Acad. Sci. U.S.A.* **80**:2026-2030 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole et al., in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)].

20 In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology [PCT/US90/02545]. In fact, according to the invention, techniques developed for the production of "chimeric antibodies" [Morrison et al., *J. Bacteriol.* **159**:870 (1984); Neuberger et al., *Nature* **312**:604-608 (1984); Takeda et al.,

Nature 314:452-454 (1985)] by splicing the genes from a mouse antibody molecule specific for p-glycoprotein together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. Such human or humanized chimeric antibodies are preferred for use in therapy of human diseases or disorders (described *infra*), since the human or humanized antibodies are much less likely than xenogenic antibodies to induce an immune response, in particular an allergic response, themselves.

According to the invention, techniques described for the production of single chain antibodies [U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778] can be adapted to produce p-glycoprotein-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries [Huse et al., *Science* 246:1275-1281 (1989)] to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for p-glycoprotein.

In a second, and preferred, embodiment of the present invention, administration of agents to inhibit dendritic cell migration are provided which are known to reverse multiple drug resistance. Suitable agents may be selected from compounds that inhibit this drug transporter, including such non-limiting examples as calcium channel blockers such as verapamil, diltiazem, dihydropyridine, nifedipine, bepridil, and nicardipine; other cardiovascular drugs, including amiodarone, dipyridamole, and quinidine; antimalarial compounds such as quinine, quinacrine, and cinchonine; cephalosporin and other antibiotics such as cefoperazone, ceftriaxone, erythromycin, valinomycin, bafilomycin, and gramicidin; cyclosporins such as cyclosporine A and staurosporine; phenothiazines such as

trifluoperazine, chlorpromazine and fluphenazine; hormones such as tamoxifen, N-desmethyldoxifen, 4-hydroxytamoxifen, toremifene, and progesterone, and other compounds such as terfenadine, monensin, yohimbine, reserpine, cremophor, benzquinamide, estramustine, and tacrolimus.

- 5 In a preferred embodiment, more potent inhibitors of multi-drug resistance than those described above are used in the practice of the present invention. Non-limiting examples include the cyclosporine analog PSC 833, MK 571, and the calcium channel blockers dexverapamil and dextropropofol. PSC 833 is a cyclosporine analog with greater binding specificity for p-glycoprotein (36); it is 10-fold more potent than cyclosporine A and can
- 10 reverse multi-drug resistance in vitro at concentrations of 0.5 to 2.0  $\mu\text{mol/L}$ . MK 571 is a substrate that antagonizes p-glycoprotein and murine multidrug resistance related protein (MRP), and is a particularly potent inhibitor of the latter. MK 571 is known to be an antagonist of the leukotriene D<sub>4</sub> receptor (54), and as will be seen in the Examples below, has utility for the purposes described herein. Other potent inhibitors of p-glycoprotein include
- 15 S9788, rapamycin, MK 571, GF120918, and SR33557.

In a further embodiment, inhibition of p-glycoprotein in dendritic cells may be achieved by targeting gene expression with antisense oligonucleotides. This approach has been demonstrated feasible in vitro using mouse 3T3 fibroblasts (41).

- As described above, another dendritic cell surface protein that may be antagonized to achieve
- 20 the practice of the present invention is tissue factor (abbreviated TF). Tissue factor is a procoagulant protein present on the surface of numerous cell types, including endothelial

cells, which line the vasculature, and monocytes, which circulate in blood. Increased expression of tissue factor by these cell types has been linked to many thrombotic disorders and pathologic states (see below with regard to increased expression). The majority of monocyte membrane-associated tissue factor is not in an enzymatically active form that can  
5 initiate clotting; in order to become active, tissue factor must form an active complex with another of the clotting factors, Factor VII or its activated form, Factor VIIa. This complex then leads to clot formation.

Also as described above in the instance of antagonists of p-glycoprotein, tissue factor may also be antagonized in order to inhibit dendritic cell mobility. This may be achieved by the  
10 use of antibodies and fragments against tissue factor. A monoclonal antibody against tissue factor that inhibits migration of DC is VIC7. Compounds known to inhibit tissue factor activity include the dilazep (42) and retinoic acid (43). In a further embodiment, inhibition of tissue factor expression in dendritic cells may be achieved by targeting gene expression with antisense oligonucleotides to tissue factor (49). A further strategy on interfering with tissue  
15 factor activity in order to suppress dendritic cell migration is through the use of purified, nonlipidated fragments of soluble recombinant human TF. Migration was inhibited in the presence of a soluble, recombinant form of human TF representing the complete amino acid sequence of the extracellular region or fragments thereof, expressed in *E. coli* and used in delipidated form. Soluble TF impeded migration by  $69 \pm 2\%$  in eight independent  
20 experiments when used at 5-20  $\mu\text{g/ml}$ . Only fragments containing amino acid residues that are carboxyl to residue 202 blocked reverse transmigration effectively. This finding agrees well with the location of the epitope for mAb VIC7, which is between amino acids 181-214.

The finding that both anti-TF mAb and soluble TF inhibit reverse transmigration is consistent with a model in which the binding of endogenous TF on monocytes to an essential ligand is prevented by anti-TF mAb and is competed with by soluble recombinant TF. The competitive binding sites contained in the region spanning 202-219.

- 5 Enhancing the effectiveness of vaccination and other forms of immunotherapy is another feature of the present invention. This aspect is carried out using methods and agents which enhance or activate the dendritic cell membrane proteins p-glycoprotein and tissue factor. Agents useful for this utility include activators of p-glycoprotein, such as bromocriptine (44), N-acetyl-leucine<sub>n</sub>-tyrosine amide where n=1-6 (45), and cytarabine (46) are preferred.
- 10 Suitable activators of tissue factor include endotoxin (47), silver ion (47), and N,N'-dimethyl- $\gamma,\gamma'$ -dipyridylum dichloride (48). Preferably, these agents are admixed with the immunogen to be used for vaccination.

- A further utility of one aspect of the present invention is in the immunotherapeutic treatment of cancer. Numerous studies and trials involve attempts to initiate or enhance an immune
- 15 response in the patient toward the patient's own tumor. However, these regimens have provided little positive results, often because the patient is unable to mount an effective immune response to the tumor antigens. In this case, effective antigen presentation maybe suppressed. The methods and agents of the present invention directed towards the enhancement of the immune response and increasing the effectiveness of vaccines has
- 20 particular utility in the enhancement of therapeutic vaccines for cancer.

In another embodiment of the present invention, methods and agents may be used to increase dendritic cell migration in order to achieve an enhancement of the development of immunity or in a subsequent immune response to an antigen. This would be carried out using agents that increase transport of endogenous substrates for p-glycoprotein that mediate dendritic cell migration. Enhanced development of immunity is particularly useful in enhancing the effectiveness of a vaccine, such as that for viruses such as poliomyelitis, rubella, measles, variola, and varicella, and especially poorly immunogenic immunogens such as influenza; bacteria such as *Borrelia burgdorferi*, the causative pathogen of Lyme disease, and other organisms including parasites such as malaria. Furthermore, the invention herein is also useful in the various newer immunotherapeutic approaches to the prevention and control of other infectious diseases such as HIV and hepatitis, and immunotherapies of various cancers (infra). In these approaches, potentially immunogenic components of the virion or tumor are prepared by various means and introduced into the patient in an attempt to develop immune recognition of the pathologic antigen(s). Often, the patient is immunocompromised and cannot mount an effective immune response to the antigen. One embodiment of the methods and agents of the present invention provide a means for increasing the delivery of antigen to the immune system by increasing or enhancing the migration of dendritic cells which have processed the antigen to the lymphatic vessels, where they may subsequently encounter T lymphocytes and induce cellular and humoral immunity.

The route of delivery of the agents of the present invention may be selected by one skilled in the art to provide the optimal contact with dendritic cells involved in the etiology of the condition to be treated or the route of exposure of the immunogen. As examples, contact dermatitis, psoriasis, and other topical conditions may be treated by application of the agent

of the present invention to the skin. Allergic conjunctivitis may be treated by application of the agent to the eye. Parenteral or other routes of administration which results in generalized delivery of the agent is also provided. Bronchiolar and pulmonary exposure may be achieved by aerosol and fine powder inhalation, respectively.

- 5 In yet another aspect of the present invention, provided herein are pharmaceutical compositions of the above agents. Such pharmaceutical compositions may be for administration for injection, or for oral, pulmonary, nasal or other forms of administration. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of a low molecular weight component or components, or derivative
- 10 products, of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (*e.g.*, Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol)
- 15 and bulking substances (*e.g.*, lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hylauronic acid may also be used. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and derivatives. *See, e.g.*, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack
- 20 Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form.

Contemplated for use herein are oral solid dosage forms, which are described generally in Remington's Pharmaceutical Sciences, 18th Ed.1990 (Mack Publishing Co. Easton PA 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid  
5 encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (e.g., U.S. Patent No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, K. In: *Modern Pharmaceutics* Edited by G.S. Banker and C.T. Rhodes Chapter 10,  
10 1979, herein incorporated by reference. In general, the formulation will include the component or components (or chemically modified forms thereof) and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

In a further embodiment of the present invention, methods and agents are provided for the  
15 augmentation of the migration of monocytes, by enhancing the functioning of the monocyte membrane protein p-glycoprotein. The selection of suitable agents and routes of delivery are as those described above in relation to modulating the same membrane protein on dendritic cells. This aspect of the invention has utility in the treatment of various disorders, preferably chronic inflammatory conditions which are not necessarily, but may be, initiated or  
20 promulgated by antigens and antigen-specific immune cells such as cytotoxic T lymphocytes. For example, the development and progression of atherosclerotic lesions involves the progressive accumulation and retention of monocytes (which become tissue macrophages) and other cell types in lesions; the local inflammation mediated by their secretory products

which attracts more pro-inflammatory cells, together with secretion of growth factors, is a pathophysiologic basis for vessel occlusion.

The methods and agents of the present invention directed to increasing monocyte migration has particular utility in preventing the retention of monocytes in inflammatory sites within the body, thus limiting the extent of inflammation. Other examples of diseases and condition in which accumulation and retention of monocytes and resultant production of inflammatory mediators, growth factors and chemoattractants for additional inflammatory cells include rheumatoid arthritis and the granulomatous diseases.

It is a further object of the present invention to provide methods for identifying agents and compound useful in modulating the migration of dendritic cells. As a consequence of the discovery by the inventors herein of the presence of p-glycoprotein on the membrane of dendritic cells and the further discovery of the role of p-glycoprotein and tissue factor in dendritic cell migration, various known methods for measuring the effect of compounds on p-glycoprotein activity may be adapted for use on dendritic cells. For example, dendritic cells may be isolated from skin explants and loaded with a dye known to be exported by functioning p-glycoprotein (28), such as 3,3'-diethyloxacarbocyanine iodide (DiOC<sub>2</sub>). Export of the dye may be measured in the absence and presence of compounds suspected of inhibiting or enhancing the activity of p-glycoprotein by decreasing or increasing, respectively, the pumping of the dye out of the cell. In a further embodiment, agents useful for the modulation of dendritic cell migration may be identified by first identifying whether a candidate agent is capable of interacting with p-glycoprotein or tissue factor; and subsequently determining the extent of modulation of the agent on the migration of dendritic

cells in vitro or in vivo. The initial screen may be performed on cells which express p-glycoprotein, such as any one of numerous cancer cell lines.

Subsequently, the effect of the compound on the migration of dendritic cells may be performed as described below or that described in U.S. Patent 5,627,025, incorporated herein  
5 by reference. This method employs human skin explants in culture to measure the effect of compounds on dendritic cell emigrating from the skin explant into the culture medium. The methods disclosed herein are not limited to any particular method but are based upon the newly-identified role of p-glycoprotein and tissue factor on dendritic cells in cell migration.

Likewise, assays may be performed to identify compounds which enhance migration of  
10 monocytes out of tissues. As one non-limiting example, migratory behavior of monocytes may be evaluated in an in vitro model of the blood vessel wall consisting of human umbilical vein endothelial cells (HUVEC) grown on type I collagen gels (see Example I, below). Human blood monocytes, which express p-glycoprotein, transmigrate across the confluent endothelium and enter the collagenous substrate. The extent of migration is measured after  
15 exposure of the monocytes to the candidate. Other methods of measuring the migration of monocytes are known to the skilled artisan and are useful in the practice of the present invention.

In a further example of a method for identifying compounds useful for modulating dendritic cell p-glycoprotein activity, the following steps are carried out:

- 20                   i)       loading isolated dendritic cells with a detectable substrate of the p-glycoprotein transporter activity;

- ii) exposing the loaded cells to an agent suspected of modulating p-glycoprotein activity;
- iii) measuring the rate of transport of the detectable substrate from the dendritic cells; and
- 5 iv) comparing said rate to the rate of p-glycoprotein transporter activity determined of control dendritic cells not exposed to the agent.

An increase or decreased in the transport rate in the presence of the agent indicates the extent of activity of the agent in increasing or decreasing, respectively, p-glycoprotein activity of said dendritic cells. In a further step, compounds that are active in the above assay, either at increasing or decreasing p-glycoprotein activity, can be additionally assays to determine if they are useful for modulating the migration of dendritic cells by determining the extent of modulation of the agent of the migration of dendritic cells in vitro or in vivo. DC migration may be determined by any of several techniques, such as the emigration rate described in aforementioned U.S. Patent 5,627,025. In another method, compounds may be evaluated directly in the emigration assay (7). Activity may be evaluated in vivo by administration of the agent to an animal, followed by, for example, application of an antigen to the skin which would under normal circumstances induce immunity in the animal or an immune response. The extent of the any reduction in the immune response elicited after administration of the agent is an indication of the extent of inhibition of dendritic cell migration. Similarly, compounds to be evaluated for the enhancement of the immune response may be evaluated in vivo by administering the agent parenterally or to the skin, for example, followed by application of an immunogen. Any resulting enhancement of the immune response as compared with control animals indicates positive activity in enhancing the immune response.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

## MATERIALS AND METHODS

5     *Transendothelial Migration Assays* Using a method detailed previously (10, 11), freshly isolated peripheral blood mononuclear cells (PBMC), approximately one-fourth of which are monocytes, were added to confluent monolayers of unstimulated HUVEC grown on type I bovine collagen gels with or without addition of mAb. Cultures were incubated for 1.5 hr; during this time, monocytes, but very few lymphocytes, transmigrate into the collagen (10).  
10    To examine their subsequent basal-to-apical transendothelial migration, monocytes were allowed to accumulate beneath the endothelium in the absence of added mAb. Then monolayers were washed twice in Medium 199 (Life Technologies, Grand Island, NY) to remove nonadherent cells from the apical surface, and individual wells received aliquots of Medium 199 containing 20% heat-inactivated human serum with or without added  
15    monoclonal antibodies (mAbs) as indicated. After 24 hr of incubation, cultures were washed twice in Medium 199 and control medium or medium containing mAb was replenished. After a total time of 48 hr in culture, samples were fixed for microscopic analysis. Differential interference contrast optics were used to quantitate the number of mononuclear phagocytes (MP) that were beneath the endothelial monolayer (11) in at least five high-power  
20    fields per sample. Each experiment was conducted with six replicates per parameter tested.

*Skin Cultures* Human split-thickness skin was obtained from the New York Firefighter's Skin Bank (New York Hospital-Cornell Medical Center) from cadavers within 24 hr of death, or

from patients undergoing plastic surgery, and was authorized for use in research. Generally, dermatomes were approximately 300-mm thick, including both epidermis (about 100-mm) and a portion of the dermis. Skin was prepared, cultured, and emigrated cells were quantitated as previously described (7). Each explant was trimmed to 400-mm<sup>2</sup> and floated in 3 to 6 ml culture medium. When used, mAbs or verapamil (Sigma) were added to the culture medium at the onset of culture, and cultures were incubated undisturbed until the indicated day of analysis. Verapamil was prepared as a concentrated stock in methanol. Final methanol concentration in cultures was 0.03% (vol/vol).

*Immunostaining and Flow Cytometry* Skin explants were separated into epidermal and dermal sheets by treatment with 0.5 M ammonium thiocyanate for 20 minutes at 37° C (12). Alternatively, skin samples were snap-frozen in OCT compound (Miles; Elkhart, IN) and then used for the preparation of thick sections (approximately 100  $\mu$ m) cut parallel to the epidermis (6). After either procedure, sheets of skin were fixed in acetone for 5 min. before staining. Sheets of epidermis or thick sections were incubated overnight at 4°C in Eppendorf tubes with CY3-conjugated goat anti-mouse IgG (1:200; Jackson Immunoresearch Laboratories, Inc.), 1:20 FITC-conjugated anti-MHC II (Becton Dickinson), or anti-MHC II 9.3C9 hybridoma supernatant. Tissues were washed three times in PBS containing 1% Tween 20, 15 min. each at room temperature. For detection of 9.3C9 when it was used, CY3-conjugated goat anti-mouse IgG (Jackson Immunoresearch, Inc.) was added for 2 hr at 4° C, followed by three washes as just described. Staining for flow cytometry was carried out as previously detailed (7).

In some experiments, explants were incubated in dispase II (Boehringer Mannheim) for 45 min. at 37°C to separate the epidermis from dermis. Pieces of epidermis were submerged in 0.05% trypsin/0.53 mM EDTA for 45 min. Single cell suspensions were generated by gently pressing epidermal pieces through a fine mesh. Aliquots of digested cells were incubated with FITC-labeled anti-MHC II for 30 min. on ice, washed twice, and analyzed by flow cytometry.

*Immunoblots* Emigrated DC were purified by negative selection: anti-CD3 mAb was incubated with emigrated cells, and T lymphocytes were removed using anti-mouse IgG-conjugated magnetic beads (Dynal). The purity of the resulting population was verified by flow cytometric evaluation after staining with PE-conjugated anti-MHC II (Becton Dickinson). Microsomal membranes from 5 million thus purified DC, 25 million PBMC, or 5 million HUVEC were prepared as described (13), subjected to SDS-PAGE (4-12% gradient gel) under reducing conditions, and electroblotted onto a nitrocellulose membrane. The membrane was probed with 5 mg/ml C219 anti-MDR-1 mAb (Centocor) and visualized using peroxidase-conjugated anti-mouse IgG, followed by ECL substrate (Amersham).

*Efflux Assays* A previously published method to detect functional MDR-1 (14) was employed with minor modifications. Emigrated cells were incubated in RPMI-1640 containing 20 ng/ml DiOC<sub>2</sub> (Molecular Probes) for 15 min. at 37 °C, washed twice in ice cold medium, resuspended in 10% fetal bovine serum/RPMI-1640 containing no additives or with addition of 10 mg/ml UIC2 mAb (Coulter). After 90 min. of incubation at 37 °C, cells were washed and immediately placed on ice for labeling with PE-conjugated anti-MHC II mAb and analysis by flow cytometry.

**EXAMPLE 1:        Role of MDR-1 in basal-to-apical transendothelial migration of mononuclear phagocytes.**

The present inventors were drawn to investigate the role of p-glycoprotein in dendritic cell migration by a set of observations with blood monocytes, now known to be a progenitor of DC when appropriately stimulated with cytokines (15-19). In these initial experiments, the migratory behavior of monocytes was examined in an in vitro model of the blood vessel wall consisting of human umbilical vein endothelial cells (HUVEC) grown on type I collagen gels (10, 11). Human blood monocytes, which express MDR-1 (20, and data not shown), transmigrate across the confluent endothelium and enter the collagenous substrate. This initial migration into the collagen, which is completed within one hr, was unaffected by the addition of anti-MDR-1 mAb, but was partially blocked by mAbs against the  $\beta_2$  chain of integrins (CD18) and CD31, as described (10, 11) (Fig. 1A).

After a brief residence in the collagen, a majority of subendothelial MP retransverse the overlying, intact endothelium with a  $t_{1/2}$  of 24 to 48 hr (21). This reverse transendothelial migration was not prevented by mAb to CD18 or CD31 (Fig. 1B). However, screening of mAbs (> 100) submitted to the Vth International Workshop for Human Leukocyte Differentiation Antigens revealed that neutralizing mAbs to MDR-1 strongly inhibited reverse transmigration. UIC2 (22), 4E3 (23), and MRK16 (24) against extracellular domains of MDR-1 inhibited reverse transmigration by 50%, 64%, and 88%, respectively (Fig. 1B). MRK16, used here as F(ab')<sub>2</sub> fragments, was the most potent blocking mAb, exhibiting maximal levels of inhibition using as little as 0.5 to 1.0 mg/ml. C219 (25), which binds an intracellular epitope of MDR-1, had no effect. Preincubation of monocytes with MRK16,

and subsequent removal of unbound mAb before addition of these cells to the endothelium blocked reverse transmigration as well as when the mAb was present continuously during the assay (Pre-MRK16, Fig. 1B). No other mAbs screened inhibited reverse transmigration, except one against tissue factor.

5     **EXAMPLE 2:       Role of MDR-1 in mobilization of DC and T lymphocytes from skin.**

During reverse transmigration, MP migrate across endothelium in a basal-to-apical direction, a movement that is reminiscent of the normal trafficking of DC from the periphery into afferent lymphatics. A panel of mAbs, including those against MDR-1, was applied to  
10     cultured human skin explants. Appearance of DC and T lymphocytes in the culture medium of the explants was reduced by  $71 \pm 9\%$ , and  $79 \pm 16\%$  when the skin was incubated in the presence of MRK16 at concentrations of 2 and 10 mg/ml, respectively (Fig. 2A). Verapamil, a drug that antagonizes MDR-1 transport (26), reduced DC and T lymphocyte accumulation in the culture medium by  $52 \pm 19\%$  at 10 mg/ml (20 mM) (Fig. 2A). The anti-MDR-1 mAb  
15     UIC2 also blocked migration, by  $75 \pm 2\%$  when used at 2 mg/ml (Fig. 2A). An isotype matched control mAb against cadherin 5 (Hec 1) (11) that does not react with DC or T cells had no effect. Moreover, isotype-matched mAbs against other DC surface proteins--CD18 (IB4), CD31 (hec7), and MHC I (W6/32)--also did not inhibit the migration significantly (Fig. 2B). Under all conditions, the cellular content of émigrés ranged from 40 to 60% DC,  
20     with the remaining population being T lymphocytes. Thus, antagonists of MDR-1 blocked migration of DC and T lymphocyte uniformly. Cell viability in all groups was >90%, as assessed by trypan blue exclusion.

**EXAMPLE 3: Expression of MDR-1 by DC.**

T lymphocytes are known to express MDR-1 (14, 20, 27), but its expression by DC has not been reported previously. Immunostaining for MDR-1 was observed on DC in the epidermis (Fig. 3A and C). The same cells also stained positively for the DC marker MHC II (Fig. 3B and C). No other cell types in the skin, such as keratinocytes or fibroblasts, showed positive staining for MDR-1 in these experiments. For these studies, skin was cultured in the presence of MRK16 for two days, as for migration analysis. Then staining was carried out on tangential sections prepared from snap-frozen skin, applying only anti-mouse Cy3-conjugated detection antibody. This approach illustrates not only the presence of MDR-1 on epidermal DC in situ, but also demonstrates that mAb applied during culture gains sufficient access to the epidermis. Skin incubated with isotype-matched control mAb hec 1 did not show positive staining.

Flow cytometric analysis of the cells that emigrated from control explants using double labeling with anti-MDR-1 (using either MRK16 or UIC2) and anti-MHC II mAbs demonstrated high levels of MDR-1 on all DC in some experiments (Fig. 4A) or on a majority, but not all, DC in other experiments (Fig. 4B). The fraction of DC staining positively was always greater than the fraction of positively stained subset of T lymphocytes (Fig. 4C).

Expression of authentic MDR-1 by DC was evident in two assays. To document expression of functional MDR-1 (28), DC were loaded with a synthetic, fluorescent substrate for MDR-1, DiOC<sub>2</sub>. At 37 °C, but not 4 °C, DC transported this substrate into the surrounding medium, showing a log decrease in fluorescence intensity (Fig. 4D), and this efflux was

inhibited by anti-MDR-1 mAb UIC2 (Fig. 4D). To identify MDR-1 molecules in DC, crude membranes were prepared and immunoblotted using mAb C219, another mAb that reacts with MDR-1. A broad band of approximately 170 to 180 kDa, the reported molecular weight of MDR-1 was identified (Fig. 4E). The band observed matched that seen in membranes from PBMC and was not observed in HUVEC (Fig. 4E), which do not express MDR-1.

**EXAMPLE 4: Effect of Verapamil on the DiOC<sub>2</sub> Efflux from Dendritic Cells**

Figure 5 further documents that functional p-glycoprotein is expressed by dendritic cells and that p-glycoprotein-mediated transport can be blocked by agents, particularly verapamil and a monoclonal antibody against p-glycoprotein, that are known to inhibit p-glycoprotein transport activity. As described in the Brief Description of Figure 5, dendritic cells were loaded with the p-glycoprotein synthetic substrate 3,3'-diethyloxcarbocyanine iodide (DiOC<sub>2</sub>), and flow cytometry performed as described in Example 3. Approximately 90% of the dye was transported out of the dendritic cells incubated in the absence of p-glycoprotein inhibitors, as indicated by the one-log decrease in fluorescence intensity in the FL1 channel (x-axis) (compare Panel C to D). However, transport of the dye was completely prevented in the presence of verapamil (Panel E) and the anti-p-glycoprotein monoclonal antibody UIC2 (Panel F).

**Example 5: Effect of MDR-1 antagonists on maturation and retention of DC in epidermis.**

To examine the step at which emigration of DC from the explants was inhibited by anti-MDR-1 mAb, epidermal and dermal sheets prepared from cultured skin explants were stained for MHC II (29, 30). Compared to the number present at the onset of culture (Fig.

6A, B), the number of DC remaining in the epidermis after three days of culture under control conditions was decreased by 56% (Fig. 6A, C). In explants cultured in the presence of MRK16 (Fig. 6A, D), UIC2 (Fig. 6A), or verapamil (Fig. 6A), a more limited reduction in DC density of 13%, 11%, and 25%, respectively, were observed. Thus, antagonism of MDR-1 results in retention of DC in the epidermis.

In cultures treated with anti-MDR-1 mAb MRK16, some DC retained in the epidermis appeared morphologically similar to epidermal DC before the onset of culture (compare Fig. 6B and D), but others showed evidence of maturation resembling that observed under control conditions (Fig. 6C and data not shown), including an increase in cell size and greater intensity of staining for MHC II (4, 29, 30). Flow cytometric analysis of DC enzymatically removed from the epidermis after culture in the presence of anti-MDR-1 mAb MRK16 or under control conditions indicated that the maturation of DC, which usually accompanies their mobilization, was not inhibited by anti-MDR-1, as exemplified by the up regulation of MHC II in the majority of DC in the presence or absence of MRK16 (Fig. 7). Thus, antagonism of MDR-1 strongly impedes the migration of DC, but only inconsistently or negligibly suppresses their maturation.

**EXAMPLE 6: Effect of Anti-Tissue Factor Antibodies on Emigration of Dendritic Cells and T Lymphocytes from Skin Explants**

Explants of human skin were floated in culture medium without added monoclonal antibody or in medium containing anti-tissue factor monoclonal antibody VIC7 or control antibodies against cadherin 5 or carcinoembryonic antigen (CEA). After three days of incubation, DC

and T lymphocytes that appeared in the culture medium were collected. Each experiment was conducted with 3-4 replicates per parameter tested. The number of emigrated cells recovered from individual control explants was typically  $5 \times 10^5$ . To compare data from different experiments, the mean number of emigrated cells in the absence of added  
5 monoclonal antibody in each experiment was set equal to 1.0 and relative values were obtained for the remaining data. Cumulative results from three independent experiments are shown in Figure 8. These experiments show that monoclonal antibody to tissue factor reduces the emigration of cells from skin explants by about 50%.

**EXAMPLE 7: Effect of P-glycoprotein Antagonists on Dendritic Cell Migration  
10 in Vivo**

To test the effectiveness of p-glycoprotein antagonists on dendritic cell migration in vivo, experiments were carried out in mice. First, the murine molecule analogous to p-glycoprotein in mouse DCs was identified. To do this, mouse skin explants, derived from the dorsal aspect of the ear, were cultured in a manner similar to the human skin explants  
15 (50). Dendritic cells (DCs) that migrated out of the lymphatic vessels of these explants were used in efflux transport assays and in flow cytometry. Efflux of the fluorescent substrate Fluo-3 for p-glycoprotein-related molecules was observed (Fig. 9A, left panel), and flow cytometric evaluation using specific antibodies demonstrated the expression of murine multidrug resistance related protein (MRP) (Fig. 9B). That MRP was the major p-  
20 glycoprotein-related transporter was further evident from the observation that MRP knock-out mice (51) failed to efflux the fluorescent substrate Fluo-3 (Fig. 9A, right panel).

The pharmacologic compound MK 571 is a substrate that antagonizes p-glycoprotein and MRP, but is a particularly potent inhibitor of the latter (52). Thus, MK 571 was added to cultures of human skin explants and mouse ear explants. Migration of DC was blocked by 58% and 62%, respectively (Figs. 10 A,B).

- 5 MRP knock-out mice and age-matched wild-type mice were employed in a contact sensitivity assay to determine whether MRP mediated the migration of murine DCs in vivo. Dendritic cells were analyzed for their transport of the contact sensitizer FITC to lymph nodes 24 h after its application to the skin of mice, using the contact sensitivity assay previously described (53). Dendritic cell accumulation in the lymph nodes of sensitized  
10 mice was reduced by 87% in the absence of MRP expression (Fig. 11A).

- Next, using wild-type mice, MK 571 was administered i.v. (50 mg / kg body weight) four hours prior to application of the contact sensitizer FITC to determine whether pharmacologic inhibitors of MRP and p-glycoprotein are effective in suppressing DC migration. Indeed, administration of MK 571 reduced accumulation of FITC-bearing DCs  
15 in the draining lymph node by 64% (Fig. 11B). These data indicate that pharmacologic antagonism of p-glycoprotein suppresses dendritic cell migration.

This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all respects illustrative and not restrictive, the scope of the

invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

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WHAT IS CLAIMED IS:

- 1 1. A method for decreasing the migration of dendritic cells from the peripheral tissues to  
2 the lymphatic vessels by contacting said dendritic cells with an effective amount of an  
3 agent capable of inhibiting the activity of a dendritic cell membrane protein selected  
4 from the group consisting of p-glycoprotein, tissue factor and the combination  
5 thereof.
- 1 2. The method of claim 2 wherein said agent is an antibody which binds to p-  
2 glycoprotein, an antibody fragment which binds to p-glycoprotein, an agent which  
3 blocks transport activity of p-glycoprotein, an antagonist of p-glycoprotein, or an  
4 antisense oligonucleotide to p-glycoprotein.
- 1 3. The method of claim 3 wherein said antibodies or antibody fragments which bind to  
2 p-glycoprotein are selected from the group consisting of MRK-16, UIC2, 4E3, and  
3 antibody fragments thereof which bind p-glycoprotein.
- 1 4. The method of claim 1 wherein said agent is capable of reversing multidrug  
2 resistance.
- 1 5. The method of claim 1 wherein said agent is a calcium channel blocker, reserpine,  
2 quinine, quinidine, quinacrine, cinchonine, cyclosporine and cyclosporine analogs,  
3 yohimbine, phenothiazines, tamoxifen, N-desmethyldramoxifen, 4-hydroxytamoxifen,  
4 toremifene, progesterone, cephalosporin antibiotics, erythromycin, valinomycin,

5           gramicidin, bafilomycin, amiodarone, dipyridamole, quinidine, cortisol, terfenadine,  
6           cremophor, benzquinamide, estramustine, monensin, tacrolimus, GF120918, S9788,  
7           rapamycin, MK 571, or SR33557.

1       6.     The method of claim 5 wherein said calcium channel blocker is selected from the  
2           group consisting of verapamil, diltiazem, azidopine, dihydropyridine, dexverapamil,  
3           nifedipine, bepridil, nicardipine, dextiguldipine, and combinations thereof.

1       7.     The method of claim 5 wherein said cyclosporin is cyclosporine A, staurosporine,  
2           PSC 833, and combinations thereof.

1       8.     The method of claim 5 wherein said phenothiazine is selected from the group  
2           consisting of trifluoperazine, chlorpromazine, fluphenazine, and combinations thereof.

1       9.     The method of claim 1 wherein said agent is an antagonist of tissue factor.

1       10.    The method of claim 9 wherein said agent is an antibody or an antibody fragment to  
2           tissue factor, a tissue factor antisense oligonucleotide, a fragment of tissue factor, or a  
3           recombinant fragment of tissue factor.

1       11.    The method of claim 9 wherein said agent is selected from the group consisting of  
2           diazepam and retinoic acid.

1       12.    A method for suppressing the development of immunity or an immune response in a  
2           mammal by contacting dendritic cells of said mammal with a therapeutically effective

amount of an agent capable of inhibiting the activity of a dendritic cell membrane protein selected from the group consisting of p-glycoprotein, tissue factor and the combination thereof.

13. The method of claim 12 wherein said agent is an antibody which binds to p-glycoprotein, an antibody fragment which binds to p-glycoprotein, an agent which blocks transport activity of p-glycoprotein, an antagonist of p-glycoprotein, or an antisense oligonucleotide to p-glycoprotein.

14. The method of claim 13 wherein said antibodies or antibody fragments which bind to p-glycoprotein are selected from the group consisting of MRK-16, UIC2, 4E3, and antibody fragments thereof which bind p-glycoprotein.

15. The method of claim 12 wherein said agent is capable of reversing multidrug resistance.

16. The method of claim 12 wherein said agent is a calcium channel blocker, reserpine, quinine, quinidine, quinacrine, cinchonine, cyclosporine and cyclosporine analogs, yohimbine, phenothiazines, tamoxifen, N-desmethyldamoxifen, 4-hydroxytamoxifen, toremifene, progesterone, cephalosporin antibiotics, erythromycin, valinomycin, gramicidin, bafilomycin, amiodarone, dipyridamole, quinidine, cortisol, terfenadine, cremophor, benzquinamide, estramustine, monensin, tacrolimus, GF120918, S9788, rapamycin, MK 571, or SR33557.

- 1 17. The method of claim 16 wherein said calcium channel blocker is selected from the  
2 group consisting of verapamil, diltiazem, azidopine, dihydropyridine, dexverapamil,  
3 nifedipine, bepridil, nicardipine, dextiguldipine, and combinations thereof.
- 1 18. The method of claim 16 wherein said cyclosporin is cyclosporine A, staurosporine,  
2 PSC 833, and combinations thereof.
- 1 19. The method of claim 16 wherein said phenothiazine is selected from the group  
2 consisting of trifluoperazine, chlorpromazine, fluphenazine, and combinations thereof.
- 1 20. The method of claim 12 wherein said agent is an antagonist of tissue factor.
- 1 21. The method of claim 20 wherein said agent is an antibody or an antibody fragment to  
2 tissue factor, a tissue factor antisense oligonucleotide, a fragment of tissue factor, or a  
3 recombinant fragment of tissue factor.
- 1 22. The method of claim 20 wherein said agent is selected from the group consisting of  
2 dilazep and retinoic acid.
- 1 23. The method of claim 12 wherein said suppression of immunity or the immune  
2 response inhibits the immune response to an allergen.

- 1     24.     The method of claim 12 wherein said suppression of immunity or the immune  
2             response is used for the treatment of organ transplant rejection, graft-vs.-host disease,  
3             autoimmune diseases, atopic diseases, or HIV infection.
- 1     25.     The method of claim 24 wherein said autoimmune diseases is rheumatoid arthritis or  
2             psoriasis.
- 1     26.     The method of claim 24 wherein said atopic disease is contact dermatitis, food  
2             allergy, allergic rhinitis, allergic conjunctivitis, asthma, or eczema.
- 1     27.     The method of claim 12 wherein said agent is administered by the topical, parenteral,  
2             ophthalmic, nasal, rectal, bronchiolar or pulmonary route.
- 1     28.     The method of claim 27 wherein said parenteral administration intravenous,  
2             intraarterial, intramuscular, subcutaneous, or intradermal.
- 1     29.     A method for increasing the migration of dendritic cells from the peripheral tissues to  
2             the lymphatic vessels by contacting said dendritic cells with an effective amount of an  
3             agent capable of increasing the activity of a dendritic cell membrane protein selected  
4             from the group consisting of p-glycoprotein, tissue factor, and the combination  
5             thereof.
- 1     30.     The method of claim 29 wherein said agent is a p-glycoprotein agonist or a tissue  
2             factor agonist.

- 1     31.    The method of claim 30 wherein said agent is bromocriptine, N-acetyl-leucine,-  
2           tyrosine amide where n=1-6, cytarabine, endotoxin, silver ion, or N,N'-dimethyl- $\gamma,\gamma'$ -  
3           dipyridylum dichloride.
- 1     32.    The method of claim 29 wherein said agent is co-administered with an immunogen to  
2           enhance the development of immunity to said immunogen.
- 1     33.    The method of claim 32 wherein said immunogen is a vaccine.
- 1     34.    A method for enhancing the development of immunity or an immune response in a  
2           mammal by contacting dendritic cells of said mammal with a therapeutically effective  
3           amount of an agent capable of increasing the activity of a dendritic cell membrane  
4           protein selected from the group consisting of p-glycoprotein, tissue factor, and the  
5           combination thereof.
- 1     35.    The method of claim 34 wherein said agent is a p-glycoprotein agonist or a tissue  
2           factor agonist.
- 1     36.    The method of claim 34 wherein said agent is bromocriptine, N-acetyl-leucine,-  
2           tyrosine amide where n=1-6, cytarabine, endotoxin, silver ion, or N,N'-dimethyl- $\gamma,\gamma'$ -  
3           dipyridylum dichloride.

1 37. The method of claim 34 wherein said agent is co-administered with an immunogen to  
2 enhance the development of immunity to said immunogen.

1 38. The method of claim 37 wherein said immunogen is a vaccine.

2 39. The method of claim 38 wherein said vaccine comprises a viral, bacterial, or parasite  
3 immunogen.

1 40. The method of claim 37 wherein said immunogen is a tumor antigen.

1 41. A method for the identification of agents useful for the modulation of dendritic cell  
2 migration comprising the steps of:

- 3 i) identifying an agent capable of interacting with the a dendritic cell  
4 membrane protein selected from the group consisting of p-glycoprotein  
5 and tissue factor; and  
6 ii) determining the extent of modulation of said agent of step (i) in the  
7 migration of dendritic cells in vitro or in vivo.

1 42. A method for the identification of agents useful for modulating the development of  
2 immunity or an immune response in accordance with the method of claim 41.

1 43. A method for identifying compounds useful for modulating dendritic cell p-  
2 glycoprotein activity comprising the steps of:

- 3           i)     loading isolated dendritic cells with a detectable substrate of the p-  
4                 glycoprotein transporter activity;  
5           ii)    exposing said loaded cells to an agent suspected of modulating p-  
6                 glycoprotein activity;  
7           iii)   measuring the rate of transport of said detectable substrate from said  
8                 dendritic cells; and  
9           iv)    comparing said rate to the rate of p-glycoprotein transporter activity  
10                determined of control dendritic cells not exposed to said agent,  
11       wherein an increase or decreased in said rate in the presence of the agent indicates the  
12       extent of activity of said agent in increasing or decreasing, respectively, p-  
13       glycoprotein activity of said dendritic cells.

1     44.   A method for identifying compounds useful for modulating the migration of dendritic  
2       cells by carrying out the method of claim 43, followed by determining the extent of  
3       modulation of said agent of the migration of dendritic cells in vitro or in vivo.

1     45.   A method for augmenting the migration of monocytes and monocyte-derived cells  
2       from tissues by contacting said monocytes with an effective amount of an agent  
3       capable of augmenting monocyte p-glycoprotein activity.

1     46.   A method of treating a chronic inflammatory disease or condition in a mammal by  
2       enhancing the migration of monocytes and monocyte-derived cells from the affected  
3       site of said disease or condition by administration of a therapeutically effective  
4       amount of an agent capable of augmenting monocyte p-glycoprotein activity.

- 1        47.    The method of claim 46 wherein said inflammatory disease or condition is  
2        atherosclerosis, rheumatoid arthritis, or granulomatous diseases.

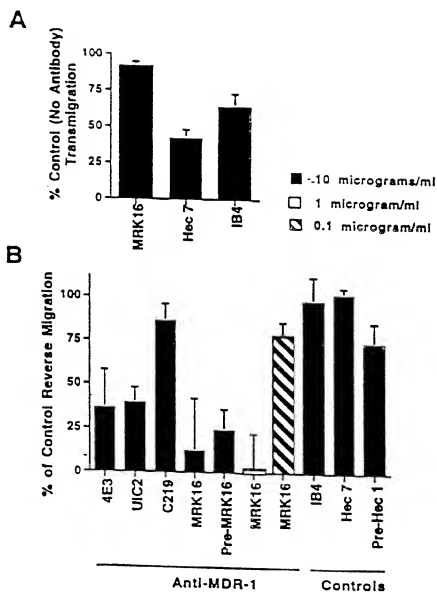


Figure 1

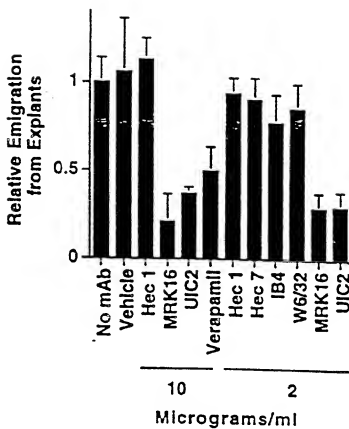


Figure 2

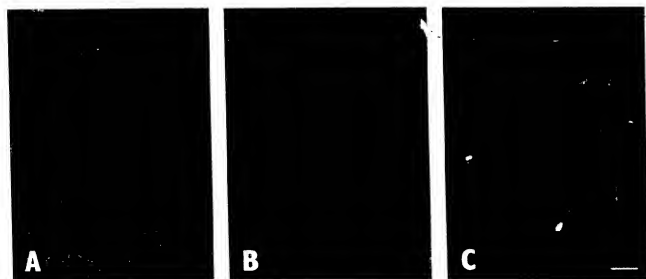


Figure 3

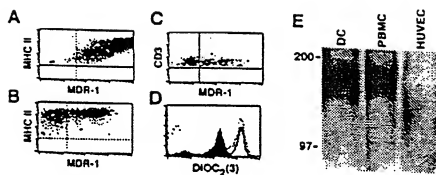


Figure 4

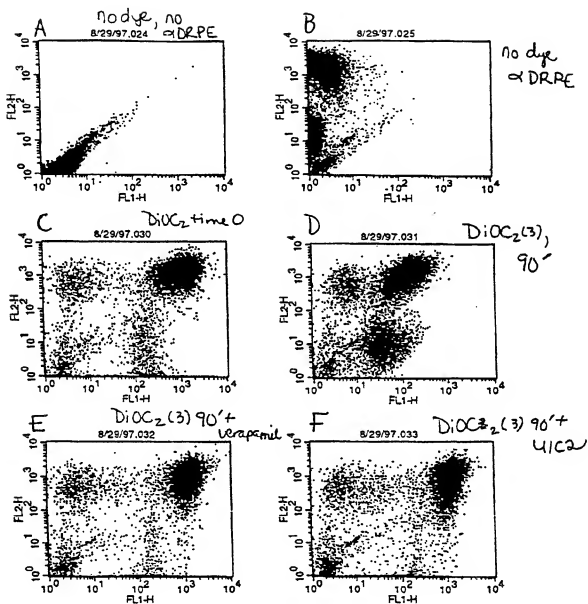


Figure 5

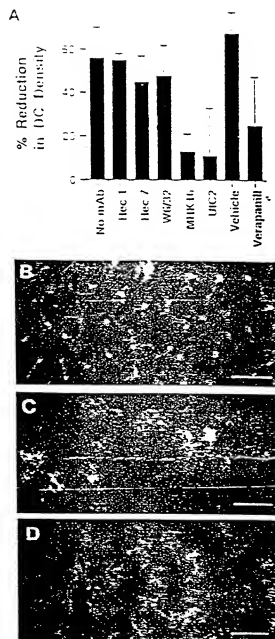


Figure 6

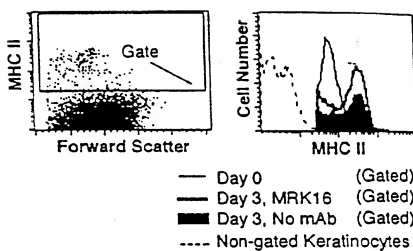


Figure 7

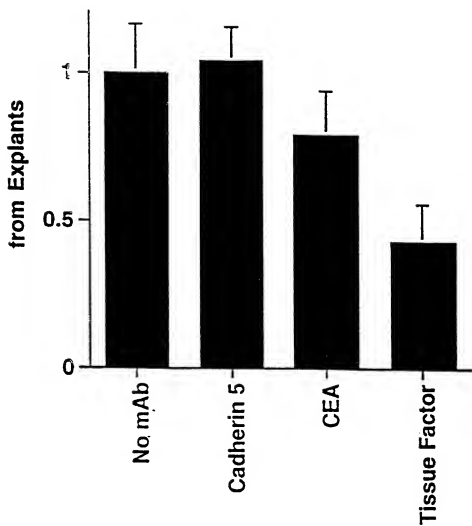


Figure 8

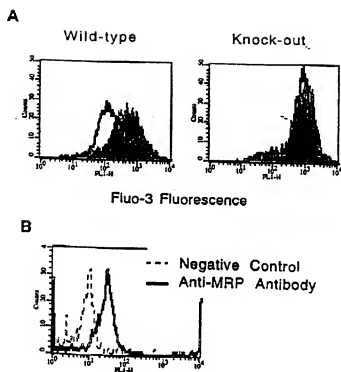


Figure 9

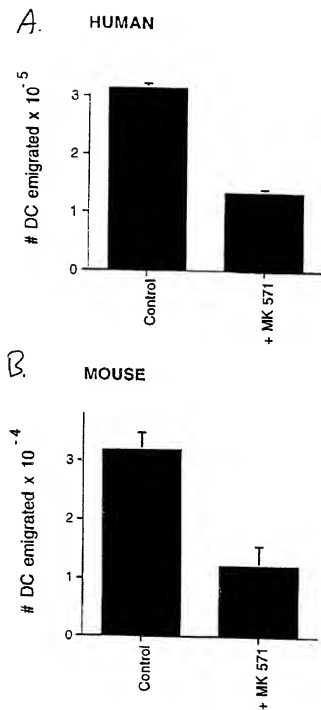


Figure 10

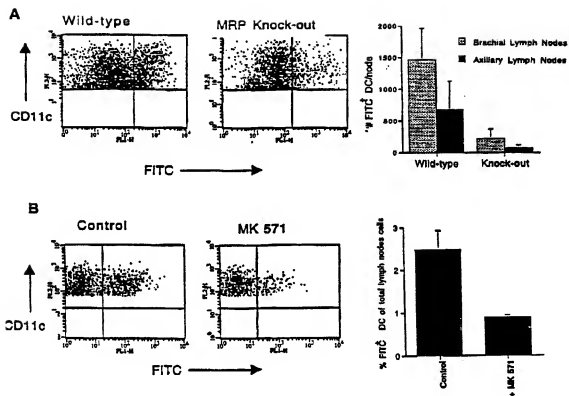


Figure 11

# INTERNATIONAL SEARCH REPORT

Inter: 1st Application No

PCT/US 99/12681

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6	A61K38/00	A61K39/395	C12N15/11	G01N33/53	A61K39/39
	A61K38/13	A61K38/36	A61K31/20	A61K31/275	C07D279/00
	A61K31/55	A61K39/00	A61K39/002	A61K39/12	A61K39/02

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>RANDOLPH G J ET AL: "Role of tissue factor in adhesion of mononuclear phagocytes to and trafficking through endothelium in vitro."</p> <p>BLOOD, (1998 DEC 1) 92 (11) 4167-77. , XP002112753</p> <p>abstract</p> <p>page 4167, left-hand column, paragraph 3</p> <p>- right-hand column, paragraph 1</p> <p>page 4169, right-hand column, paragraph 2</p> <p>- page 4170, right-hand column, paragraph 3</p> <p>page 4172, right-hand column, paragraph 2</p> <p>- left-hand column, paragraph 4</p> <p>page 4175, left-hand column, paragraph 1</p> <p>- right-hand column, paragraph 1</p> <p>page 4176, left-hand column, paragraph 1</p> <p>---</p> <p>-/-</p>	<p>1,9,10, 12,20, 21,23</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

20 August 1999

Date of mailing of the international search report

13/09/1999

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Covone, M

# INTERNATIONAL SEARCH REPORT

Intern. Appl. No.

PCT/US 99/12681

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 //C07K14/705,C07K14/745,C07K16/36,C07K16/28

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 12633 A (IMMUNEX CORP) 10 April 1997 (1997-04-10) page 2, line 31 - page 3, line 8 page 9, line 29-34 claims 16-19 ---	29-40, 44,45
A	CUMBERBATCH M ET AL: "Tumour necrosis factor-alpha is required for accumulation of dendritic cells in draining lymph nodes and for optimal contact sensitization." IMMUNOLOGY, (1995 JAN) 84 (1) 31-5. , XP002112754 page 31, left-hand column, paragraph 1 - page 32, left-hand column, paragraph 1 page 32, left-hand column, paragraph 5 page 34, left-hand column, paragraph 1 - right-hand column, paragraph 2 --- -/-	1-27, 41-44, 46,47



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/12681

G.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 96 01645 A (PEPTECH UK LTD ;LEDGER PHILIP (GB)) 25 January 1996 (1996-01-25)</p> <p>page 1, line 6-22 page 22, line 23 - page 23, line 22 example 4</p>	1-27, 41-44, 46,47
A	<p>MA J ET AL: "In vivo treatment with anti-ICAM-1 and anti-LFA-1 antibodies inhibits contact sensitization-induced migration of epidermal Langerhans cells to regional lymph nodes." CELLULAR IMMUNOLOGY, (1994 OCT 15) 158 (2) 389-99. , XP002112755 abstract page 392, paragraph 6 - page 394, paragraph 1 page 398, paragraph 3</p>	1-47
A	<p>DATABASE MEDLINE 'Online! US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US LARSEN C P ET AL: "Migration and maturation of Langerhans cells in skin transplants and explants." retrieved from STN Database accession no. 91037811 XP002112758 abstract &amp; JOURNAL OF EXPERIMENTAL MEDICINE, (1990 NOV 1) 172 (5) 1483-93. ,</p>	1-47
A	<p>LAVIE Y. ET AL.: "Agents that reverse multidrug resistance, Tamoxifen, Verapamil, and Cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells" J. BIOL.CHEM., vol. 272, no. 3, 17 January 1997 (1997-01-17), pages 1682-87, XP002112756 abstract page 1686, paragraph 1 page 1687, paragraphs 2,3</p>	1,4-8, 12,15-19
P,X	<p>RANDOLPH G J ET AL: "A physiologic function for p - glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 JUN 9) 95 (12) 6924-9. , XP002112757 the whole document</p>	1-47

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 12681

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
1-40 and 45-47 (completely), 41-44 (partially)  
  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

Continuation of Box I.1

Although claims 41-44 (partially) are directed to a diagnostic method practised on the human/animal body and claims 1-40, 45-47 (completely) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

-----  
Continuation of Box I.1

Claims Nos.: 1-40 45-47 (completely), 41-44 (partially)

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal body

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/12681

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9712633 A	10-04-1997	AU 697539 B	08-10-1998
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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup>:</b> <b>A61K 38/00, 39/395, C12N 15/11, G01N 33/53, A61K 39/39, 38/13, 38/36, 31/20, 31/275, C07D 279/00, A61K 31/55, 39/00, 39/002, 39/12, 39/02 // C07K 14/705, 14/745, 16/36, 16/28</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/62537</b> <b>(43) International Publication Date:</b> 9 December 1999 (09.12.99)
<b>(21) International Application Number:</b> PCT/US99/12681 <b>(22) International Filing Date:</b> 4 June 1999 (04.06.99)  <b>(30) Priority Data:</b> 09/090,781           4 June 1998 (04.06.98)       US Not furnished       3 June 1999 (03.06.99)       US  <b>(71) Applicants:</b> THE ROCKEFELLER UNIVERSITY [US/US]; 1230 York Avenue, New York, NY 10021-6399 (US); THE CORNELL RESEARCH FOUNDATION [US/US]; 20 Thornwood Drive, Ithaca, NY 14850 (US).  <b>(72) Inventors:</b> BEAULIEU, Sylvie; 504 East 63rd Street, #27N, New York, NY 10021 (US). RANDOLPH, Gwendalyn, J.; 235 Cypress Lane, Westbury, NY 11590 (US). MULLER, William, A.; 35 North Bayles Avenue, Port Washington, NY 11050 (US). STEINMAN, Ralph, M.; 62 North Avenue, Westport, CT 06880 (US).  <b>(74) Agent:</b> YAMIN, Michael, A.; Klauber & Jackson, 411 Hackensack Avenue, Hackensack, NJ 07601 (US).	<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> METHODS AND AGENTS FOR MODULATING THE IMMUNE RESPONSE AND INFLAMMATION INVOLVING MONOCYTE AND DENDRITIC CELL MEMBRANE PROTEINS		
<b>(57) Abstract</b> <p>Methods and agents are provided to decrease or increase the migration of dendritic cells for the suppression or enhancement, respectively, of the development of immunity and the immune response, by modulating the dendritic cell membrane proteins p-glycoprotein (MDR-1) and tissue factor. Agents which suppress migration have utility in the treatment of immunologically-mediated and inflammatory diseases such as graft rejection, contact dermatitis, seasonal allergies, asthma, and food allergies. Agents which enhance migration are useful for increasing the effectiveness of vaccines. Agents are also disclosed which enhance the migration of monocytes, useful in the treatment of chronic inflammatory diseases. Methods are also provided for identifying useful agents by measuring the effect on dendritic cell migration of agents which modulate p-glycoprotein and tissue factor activity, as well as the effect of agents on monocyte migration.</p>		

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**METHODS AND AGENTS FOR MODULATING THE IMMUNE RESPONSE AND  
INFLAMMATION INVOLVING MONOCYTE AND DENDRITIC CELL  
MEMBRANE PROTEINS**

**RESEARCH SUPPORT**

5 The research leading to the present invention was supported in part by Public Health Service grants HL46849, AI13013, and AI40045 from the National Institutes of Health, and National Research Service Award HL09722. The government may have certain rights in the present invention.

**FIELD OF THE INVENTION**

10 The present invention relates generally to methods and agents for enhancing or suppressing the development of immunity and the immune response by modulating the migration of dendritic cells, and methods for identifying agents with migration modulating activity. Methods and agents for the enhancement of monocyte migration out of chronic inflammatory foci are also provided.

**BACKGROUND OF THE INVENTION**

15 The development of immunity to a specific antigen and the subsequent immune response on re-encountering that antigen involve multi-step interactions between several immune cell types and co-stimulatory molecules with critical spatial and temporal components. In brief, an antigen encountered by antigen-presenting cells is taken up, processed, and its peptides  
20 subsequently prime T lymphocytes to generate antigen-specific T lymphocytes (e.g.,

cytotoxic and memory cells), and through the interaction with B cells, result in the formation of antigen-specific antibodies. Immune responses are initiated after antigen-presenting cells, particularly dendritic cells (DC), capture antigens in organs such as skin, lung and gut, and migrate via afferent lymphatic vessels to draining lymph nodes. T lymphocytes continually recirculate through lymph nodes, so the newly arrived, antigen-bearing DC become positioned to select lymphocytes that bear receptors for the presented antigens (1). For example, when skin is transplanted (2), or a contact allergen such as poison ivy is applied (3), epidermal DC (also termed Langerhans cells) migrate from their epithelial locations and carry antigens or allergens to the lymph node. If the lymphatic conduits to the draining lymph nodes are severed, the immune system does not become primed to the antigen (2). As they migrate, the DC undergo maturation, increasing their expression of molecules involved in antigen presentation, including major histocompatibility complex (MHC) II products, CD80 (B7-1), and CD86 (B7-2) (4, 5).

Thus, dendritic cell migration is a critical step in both the induction of immunity and in the immune response. Dendritic cell migration is essential for the successful induction of an immune response to an administered vaccine, for eliciting antibody production and cell-mediated immunity against infectious microorganisms and arising neoplastic cells within the body. Likewise, dendritic cell migration is responsible for diseases involving an exaggerated, inappropriate or undesired immune response such as allergic asthma, food allergies, contact dermatitis, seasonal allergies, and autoimmune diseases such as psoriasis, in which known or as-yet undescribed antigens are transported to T lymphocytes.

White blood cells known as monocytes are precursors of dendritic cells and tissue macrophages. These cells migrate throughout the tissues, and may develop into dendritic cells, as described above, or macrophages, which remain in the tissues. Excessive retention and pro-inflammatory activity of macrophages in certain tissues is implicated in acute and chronic inflammatory diseases, including atherosclerosis, rheumatoid arthritis, and granulomatous diseases.

Blockage of or interference with dendritic cell migration interferes with the immune response. Blockage has been achieved by the use of antibodies against certain dendritic cell proteins. Before leaving the epidermis, dendritic cells down-regulate E-cadherin, allowing retraction from neighboring keratinocytes (31). In the presence of anti- $\alpha_6$  integrin antibody, epidermal DC up-regulate MHC II and retract from neighboring keratinocytes, and fail to migrate out of the epidermis (32). Another adhesion molecule, CD44, is also used by DC during mobilization from the epidermis (33).

It is towards improved means for specifically modulating the migration of dendritic cells from the periphery to lymphatic vessels for both the positive and negative control of the immune response, and blockage of monocyte migration to control the inflammatory response, that the present invention is directed.

The citation of any reference herein should not be deemed as an admission that such reference is available as prior art to the instant invention.

The inventors herein have made the surprising and unanticipated finding that certain dendritic cell membrane proteins, namely p-glycoprotein, also known as MDR-1, the transporter responsible for multi-drug resistance, and tissue factor (TF), a procoagulant protein, are involved in the migration of dendritic cells (DCs) from the peripheral tissues to the lymphatic vessels. Alteration of dendritic cell migration activity by manipulating these surface proteins is useful for both enhancing and suppressing the immune response. Furthermore, the migration of monocyte-derived cells out of tissues has been found to be inhibited by agents which interfere with monocyte p-glycoprotein.

Thus, in accordance with the present invention, methods for the modulation of immunity and the immune response in a mammal is provided by an agent which is capable of interacting with the dendritic cell membrane proteins p-glycoprotein and tissue factor, and altering the migration of dendritic cells from the peripheral tissues to the lymphatic vessels. In one embodiment, modulation results in decreasing the migration of dendritic cells and hence the suppression of immunity or of an immune response. Agents useful for the practice of this aspect of the invention include antibodies and antibody fragments which bind to p-glycoprotein, agents which block transport activity of p-glycoprotein, antisense oligonucleotides to p-glycoprotein and antagonists of p-glycoprotein. Preferred agents are those which reverse multi-drug resistance, such as calcium channel blockers, reserpine, certain antimalarial drugs, cyclosporins, phenothiazines, tamoxifen and its metabolites, certain antibiotics, antisense oligonucleotides, and other compounds known to antagonize the activity of p-glycoprotein. Useful antibodies include MRK-16, UIC2, 4E3, and their fragments.

In a further embodiment, antagonists of tissue factor are useful for the suppression of the development of immunity and of the immune response. Such antagonists include antibodies and antibody fragments to tissue factor, tissue factor antisense oligonucleotides, fragments of tissue factor, and compounds known to antagonize tissue factor such as dilazep and retinoic acid.

Suppression of the immune response is useful for the treatment of several diseases and conditions. For example, allergic reactions, graft-vs-host disease, transplant rejection, autoimmune diseases, progression of HIV infection, contact dermatitis, and food allergy are treatable by the methods and agents of the present invention. The agents may be administered by appropriate routes including topical, parenteral, ophthalmic, nasal, pulmonary, etc., for delivery to the site of encounter of the dendritic cells with the antigen and to prevent their subsequent migration to the lymphatic vasculature.

In a further embodiment, certain methods and agents of the present invention may be used which enhance the migration of dendritic cells from the peripheral tissues to the lymphatic vessels. These methods and agents promote the development of immunity or of an immune response to a newly encountered antigen and enhance immunity or the immune response on re-encountering an antigen. Agents useful in the practice of this aspect of the present invention are agonists of the dendritic cell membrane proteins p-glycoprotein and tissue factor. Examples of useful agents include bromocriptine, N-acetyl-leucine-leucine-tyrosine amide, and cytarabine for p-glycoprotein. In a preferred embodiment, the agent is co-administered with an immunogen to enhance the development of an immune response to said immunogen, for example, when the immunogen is a vaccine.

In a further aspect of the present invention, methods for the identification of agents useful for the modulation of dendritic cell migration is provided. These methods comprise the steps of first identifying an agent capable of interacting with the dendritic cell membrane proteins p-glycoprotein and tissue factor, followed by determining the extent of modulation of the migration of dendritic cells in vitro or in vivo by the agent. These methods identify agents useful for modulating the development of immunity or an immune response.

In another aspect of the present invention, methods for the modulation of inflammation in a mammal are provided by an agent which is capable of enhancing the function of the monocyte membrane protein p-glycoprotein, and increasing the migration of inflammatory monocytic cells from the tissues. Agents useful for the practice of this aspect of the invention include the aforementioned agonists of p-glycoprotein. Increasing monocyte migration out of tissues is useful in the treatment of such inflammatory conditions as atherosclerosis, rheumatoid arthritis and granulomatous diseases.

In a still further aspect of the present invention, methods for the identification of agents useful for the augmentation of monocyte migration out of tissues is provided. These methods comprise the steps of first identifying an agent capable of enhancing the monocyte membrane protein p-glycoprotein, followed by determining the extent of augmentation of the migration of monocytes in vitro or in vivo by the agent. These methods identify agents useful for treating inflammatory conditions and diseases.

These and other aspects of the present invention will be better appreciated by reference to the following figures and Detailed Description.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1 A-B.** Effect of anti-MDR-1 monoclonal antibodies (mAbs) on transendothelial migration of mononuclear phagocytes. (A) Monocytes were allowed to migrate across endothelial monolayers with or without addition of anti-MDR-1 mAb MRK16, or mAb to  $\beta_2$  integrin (IB4) or CD31 (hec 7). Cultures were incubated for 1.5 hr. (B) To examine subsequent basal-to-apical transmigration, monocytes were first allowed to accumulate beneath the endothelium in the absence of added mAb for 1.5 hours, followed by addition of mAbs (all IgG2a; MRK16 used as  $F(ab')_2$  fragments) at the indicated concentrations; in some samples, monocytes were preincubated with MRK16 or isotype-matched control mAb hec 1 against cadherin 5 (Pre-MRK16 and Pre-Hec 1) before addition to the endothelium. After 48 hr, cultures were fixed for analysis. Data are plotted relative to control levels of reverse transmigration (no mAb added), in which 50% of the subendothelial MP retransverse the endothelium in 48 hr. Bars represent means  $\pm$  SD from three to ten experiments, except UIC2 which was tested in a single experiment.

**Figure 2.** Effect of anti-MDR-1 mAb on emigration of DC and T lymphocytes from skin explants. Explants of human skin were floated in culture medium without added mAb ( $n = 10$ ) or in medium containing anti-MDR-1 mAbs MRK16 ( $n = 10$ ) or UIC2 ( $n = 3$ ), anti-cadherin 5 mAb hec 1 ( $n = 5$ ), anti-CD31 mAb hec 7 ( $n = 3$ ), anti-MHC I mAb W6/32 ( $n = 3$ ), anti-CD18 mAb IB4 ( $n = 2$ ), verapamil ( $n = 3$ ), or the vehicle control for verapamil, 0.03% methanol (Vehicle,  $n = 1$ ). After three days of incubation, DC and T lymphocytes that appeared in the culture medium were collected and counted as previously described (7). Each experiment was conducted in triplicate;  $n =$  number of experiments in which condition was

included. The number of emigrated cells recovered from individual control explants was typically  $5 \times 10^5$ . To compare data from different experiments, the mean number of emigrated cells in the absence of added mAb in each experiment was set equal to 1.0 and relative values were obtained for the remaining data.

5 **Figure 3 A-C.** Expression of MDR-1 in situ by DC. (A) Epidermis derived from explants cultured with anti-MDR-1 mAb MRK16 were fixed and Cy3-conjugated anti-mouse IgG was added to detect expression of MDR-1 (red). Addition of Cy3-labeled detection antibody to skin incubated in the presence of nonbinding control mAb (Hec1) showed no staining (not shown). (B) Immunostaining of same sample using FITC-conjugated anti-MHC II mAb  
10 (green) identified dendritic cells. (C) Doubly-exposed frame to examine co-localization of p-glycoprotein and MHC II on dendritic cells (yellow). Bar is 10  $\mu$ m.

**Figure 4 A-E.** Expression of functional MDR-1 by emigrated DC. (A-C) MDR-1 expression in emigrated skin cells was examined by flow cytometry using double-staining with anti-MDR-1 mAb and the DC lineage marker MHC II (A, B) or the T lymphocyte lineage  
15 marker CD3 (C). Quadrants were marked based on the level of fluorescence intensity observed in samples stained with negative control mAbs. (D) For studies measuring efflux, emigrated cells were loaded with the dye 3,3'-diethyloxycarbocyanine iodide (DiOC<sub>2</sub>) (- - -, no dye added) and cultured at 37 °C for 90 minutes without addition of mAb (filled profile), in the presence of UIC2 mAb (bold line), or kept at 4 °C for this duration (thin line).  
20 Analysis of efflux in the DC fraction of emigrants was assessed by double labeling with PE-conjugated anti-MHC II mAb for flow cytometry. Figure shown was gated on MHC II<sup>+</sup> cells. (E) Membranes prepared from purified, emigrated DC, PBMC, or HUVEC were

immunoblotted with anti-MDR-1 mAb C219. Numbers to the left of bands are molecular weight markers.

**Figure 5 A-F.** Effect of verapamil on the efflux of the p-glycoprotein synthetic substrate 3,3'-diethyloxacarbocyanine iodide ( $\text{DiOC}_2$ ) from dendritic cells. Panels A and B show the fluorescence intensity of dendritic cells before loading with  $\text{DiOC}_2$ . In these cells, the FL1 staining intensity (x-axis) is low. Panels C-F show dendritic cells incubated under various conditions after loading with  $\text{DiOC}_2$ , which fluoresces on the FL1 channel. Panels B-F show cells after labeling for the cell surface marker MHC II (FL2 channel). Cell that shift  $10^3$  staining intensity on the FL2 channel are dendritic cells. For efflux measurements, dendritic cells emigrated from skin were loaded with the dye and analyzed immediately for dye content (time 0, Panel C), or cultured for 90 minutes at  $37^\circ\text{C}$  without additives (D) or in the presence of verapamil (10  $\mu\text{g}/\text{ml}$ ) (Panel E) or the anti-p-glycoprotein mAb UIC2 (10  $\mu\text{g}/\text{ml}$ ) (Panel F).

**Figure 6 A-D.** Retention of DC in the epidermis after treatment with MDR-1 antagonists.

(A) Epidermal sheets were stained with anti-MHC II mAb to enumerate DC before the onset of culture or after three days of culture in the absence of mAb (no mAb,  $n = 7$ ), or in the presence of anti-cadherin 5 mAb hec 1 ( $n = 3$ ); anti-MHC I mAb W6/32 ( $n = 3$ ); anti-CD31 mAb hec 7 ( $n = 3$ ); anti-MDR-1 mAb MRK16 ( $n = 6$ ); anti-MDR-1 mAb UIC2 ( $n = 1$ ); verapamil ( $n = 2$ ); or the vehicle control for verapamil, 0.03% methanol (Vehicle,  $n = 1$ ).  $n =$  number of experiments in which each condition was examined. DC were counted from *en face* examinations of epidermal sheets in 16 to 20 high-power fields per experiment. Percent reduction in DC density was calculated by comparing the number of DC in cultured explants

to the mean number present in a portion of the same skin sample before culture (typically 75 cells/field). **B-D)** Photomicrographs show the distribution of DC within the epidermis before culturing of explants (**B**), after three days of culture under control conditions (no mAb) (**C**), and after three days of culture in the presence of MRK16 (**D**). Bar is 20  $\mu$ m.

- 5     **Figure 7.** Effect of anti-MDR-1 mAb on the maturation of DC. The levels of MHC II expressed by epidermal DC were analyzed in gated epidermal suspensions by flow cytometry before the onset of culture (Day 0) or after three days of culture in the absence of mAb or in the presence of MRK16. Single cell suspensions of the epidermis were prepared by digestion with dispase followed by trypsin. Keratinocytes and other skin cells were excluded from the analysis by setting a gate to include only MHC II<sup>+</sup> cells.
- 10

- Figure 8.** Effect of anti-tissue factor monoclonal antibody (mAb) on emigration of dendritic cells (DC) and T lymphocytes from skin explants. Explants of human skin were floated in culture medium without added mAb or in medium containing anti-tissue factor mAb VIC7 or control mAbs against cadherin 5 or carcinoembryonic antigen (CEA). After three days of incubation, DC and T lymphocytes that appeared in the culture medium were collected. Cumulative results from three independent experiments are shown. Each experiment was conducted with 3-4 replicates per parameter tested. The number of emigrated cells recovered from individual control explants were typically  $5 \times 10^5$ . To compare data from different experiments, the mean number of emigrated cells in the absence of added mAb in each experiment was set equal to 1.0 and relative values were obtained for the remaining data.
- 15
- 20

- Figure 9 A-B.** Analysis of expression of p-glycoprotein-related transporters in mice. A) Dendritic cells emigrated from wild-type or MRP knock-out mouse ear explants were loaded with the fluorescent transporter substrate Fluo-3 and cultured at 37° C for 60 minutes (open profile) or kept at 4° C for this duration (filled profile). Analysis of efflux in the DC fraction of emigrants was assessed by double labeling with PE-conjugated anti-MHC II mAb for flow cytometry. Figure shown was gated on MHC II<sup>+</sup> cells. B) MRP expression in emigrated skin cells was examined by flow cytometry in permeabilized cells using double-staining with anti-MRP mAb MRPr1 (Signet Laboratories) or rat control mAb R35-95 (Pharmingen), followed by FITC-conjugated anti-rat mAb for detection.
- Figure 10 A-B.** Effect of MRP antagonist MK 571 on emigration of DC from skin explants. Explants of human skin or explants of the dorsal aspect of mouse ear were floated in culture medium with or without addition of MK 571 (25 µM). Murine emigrated dendritic cells were collected and explants were transferred to fresh medium daily (with or without MK 571) and were analyzed over 5 days. Human skin explants were analyzed on day 2, with a medium change or fresh addition of drug at day 1. Each experiment was conducted using 3 (human) to 10 (mouse) replicates and was performed on at least two independent occasions.

- Figure 11 A-B.** Contact sensitization in MRP knock-out mice or after administration of MRP antagonist to wild-type mice. A) Twenty five microliters of FITC dissolved in acetone:dibutylphthalate (8 mg/ml stock) was applied to the dorsothoracic area of shaved mice. Twenty four hours later, draining lymph nodes were collected, disaggregated to

obtain single cell suspensions, and stained for the dendritic cell marker CD11c. CD11c was detected using biotinylated anti-CD11c and streptavidin-conjugated R-phycoerythrin. Data are from a single experiment performed in quadruplicate. B). Mice were injected with 1 mg MK 571 intraperitoneally before application of FITC and analysis as described in (A). Data are from 2 experiments, each performed with triplicate samples.

### DETAILED DESCRIPTION OF THE INVENTION

The following terms shall have the definitions set out herein. The terms "immunity" or "development of immunity" are used generally to refer to the initial encounter of the immune system with an antigen and the development of a specific immune response to the antigen.

10 This entails, in brief, the uptake of the antigen by antigen-presenting cells (APCs), especially dendritic cells (DC), processing of the antigen, and presentation of antigen peptides in a complex with MHC molecules, to T lymphocytes, with the subsequent formation of antigen-specific T lymphocytes, including memory, helper, and cytotoxic T lymphocytes, as well as antibody-producing B lymphocytes. The term immune response is generally used herein to

15 refer to the response of the immune system to a subsequent encounter with an antigen to which immunity has been previously established. One aspect of this encounter is the uptake of the antigen by APCs, migration to draining lymphoid organs including lymph nodes and the spleen, and subsequent presentation to memory T lymphocytes that circulate in lymphoid tissues.

20 The terms "inflammation" and "inflammatory response" refer to the acute or chronic response to a known or unknown antigen in which a particular general or local site within the body exhibits adverse sequelae of the accumulation of white blood cells, including monocytes, and

their secreted mediators, producing a range of effects of varied intensities including pain, tenderness, edema, destruction of tissue, organ failure, asthma, and urticaria (hives). In the case of atherosclerosis, a chronic inflammatory disorder of the arterial wall, accumulation and retention of monocytes in the intimal tissue of the artery leads to lipid-laden plaque formation and danger of thrombosis.

As described above, the present invention relates to methods and agents for the modulation of the immune response involving the dendritic cell membrane proteins p-glycoprotein and tissue factor. As will be seen in the examples below, these proteins have been discovered to have an important physiological role in the migration of dendritic cells from the peripheral tissues, a location where antigen is encountered, to the lymphatic vessels, where interaction of the dendritic cell with T lymphocytes induces immunity or an immune response.

Manipulation of these proteins to antagonize or agonize their activities has been found to be useful for decreasing or increasing, respectively, the ability of the dendritic cell to migrate to the lymphatic vessels and thus modulate the development of immunity or an immune response to an antigen or antigens encountered by dendritic cells.

p-Glycoprotein, also known as MDR-1, is a large plasma membrane protein that functions as an ATP-driven chemotherapeutic drug efflux pump. The endogenous substrate(s) for the p-glycoprotein transporter has not yet been identified, but based on structure-activity relationships of numerous compounds, are believed to include cationic, hydrophobic molecules with at least two planar rings and a molecular weight of 400-1500 (40). Endogenous substrates also likely include modified phospholipids, such as phospholipids that are involved in cell signaling. The presence of p-glycoprotein on cells confers multi-drug

resistance ("MDR"); in cancer cells, p-glycoprotein pumps out of the cell chemotherapeutic agents and thus renders cancer cells resistant to chemotherapy (36). Thus, to enable or improve cancer chemotherapy in resistant tumors, p-glycoprotein has been a target for pharmacologic intervention to disable the pump (36). Numerous compounds have been found which antagonize the activity of the pump, and several are in use or in clinical development as adjunct therapy with cancer chemotherapeutic agents to block resistance. Prophylactic use has also been suggested to prevent the appearance of a drug resistant phenotype.

Several gene products that are highly homologous to p-glycoprotein also confer drug resistance and can transport a similar profile of substrates as p-glycoprotein. Examples of these related proteins include cMOAT (canalicular multiple organic anion transporter), LRP (lung resistance protein), and MRP (multi-drug resistance related protein). Expression of these p-glycoprotein-related gene products in monocytes or dendritic cells of humans or other mammalian species may confer the same function as the MDR-1 gene product. Therefore, p-glycoprotein as defined herein embraces the MDR-1 gene product or the gene product(s) of structurally and functionally related transporters that mediate dendritic cell migration in humans and other mammals.

Methods and agents which have been found to effectively antagonize the activity of the p-glycoprotein protein are useful in the practice of the present invention for antagonizing p-glycoprotein and thus inhibiting dendritic cell migration and suppressing immunity or an immune response. These methods and agents comprise several approaches, including but not limited to antibodies and fragments which bind to p-glycoprotein, compounds which reverse

multi-drug resistance, and means to reduce the expression of p-glycoprotein. These methods and agents may be used singly or in combination. As will be described in more detail below, administration may be targeted to a particular location of the body where the activity is desired, for example on or in an cutaneous lesion such as psoriasis or contact dermatitis, and  
5 delivery by aerosol or powder to the bronchi or lungs in the case of asthma.

Suppression or down-regulation of immunity and the immune response has utility in the prophylaxis and treatment of numerous diseases and conditions. Methods and agents of the present invention are useful as prophylaxis against the exposure of an individual to a known immunogen or allergen against which an immune response would be highly undesirable. For  
10 example, acute exposure to a causative agent of contact dermatitis, such as might occur in an industrial setting, for example, exposure to protein-modifying metals and their salts such as nickel and chromate as a result of a chemical spill, or release of a gaseous or airborne particulate allergen; a further example is topical or pulmonary exposure to urushiol, from contact with poison ivy or inhalation of smoke from burning foliage. Immunity to accidental  
15 oral consumption of a potent immunogen may be blocked by the methods and agents of the present invention. An individual exposed under such conditions can prevent the delivery of the antigen to the lymph nodes by the methods and agents of the present invention.

Wider utility of the methods and agents of the present invention are found in the treatment of acute and chronic conditions mediated by the initial or repeat exposure of the immune system  
20 to a known or as-yet unidentified antigen causative of a condition or disease. A role for dendritic cells in the development of immunity has been established; the role of dendritic cells in the subsequent immune response has been established for many antigens and is

suspected in others. The agents of the present invention that suppress the migration of dendritic cells are useful for one or both of these aspects, and especially in the type of immune response known as delayed-type hypersensitivity and more particularly, contact hypersensitivity. Examples of known immunogens that cause disease include pollen and other airborne allergens which cause allergic rhinitis and other seasonal allergic reactions, hay fever, allergic conjunctivitis; arthropod-borne antigens such as dust mites and insect secretions such as those that cause the reaction to mosquito bites; foods that cause allergies, etc. In numerous autoimmune diseases, suspected antigens to which an immune response is responsible for the pathology have been identified; in others, the antigens are probable, and in others, they are uncertain. Examples of as-yet identified immunogens responsible for pathological inflammatory conditions include food allergies of unknown cause, irritable bowel syndrome, rheumatoid arthritis, psoriasis and a large number of autoimmune diseases which are believed to be initiated and maintained by endogenous antigens. In a further utility of the present invention, means to block an immune response to transplanted antigens, in the form of acute rejection, is provided. This may be used as an adjunct to immunosuppressive and other agents routinely used in transplantation.

The methods and agents of the present invention are not limited to, but are particularly useful in, the treatment of inflammatory conditions where the acute or chronic exposure to antigen occurs in the periphery, such as the skin, as well as the gut and the lungs.

As mentioned above, one particular condition amenable to treatment by the methods and agents of the present invention is in the area of transplantation. A critical role has been identified in the interaction of antigen-presenting cells, dendritic cells in particular, with T

lymphocytes in initiating rejection. It is well established that dendritic cells mediate allograft rejection, since experimental techniques used to deplete allografts of endogenous dendritic cells do not give rise to subsequent rejection responses in recipients. However, it is not clinically feasible to deplete allografts of endogenous dendritic cells. Because it is the migration of dendritic cells to the graft recipient's lymph nodes and spleen that ultimately results in graft rejection, clinically feasible methods to block dendritic cell migration to these lymphoid tissues would prevent graft rejection. Suppression of the migration of dendritic cells by agents which antagonize p-glycoprotein or tissue factor and thus inhibit their interaction with T lymphocytes blocks the development of the immune response and thus are useful for averting graft rejection.

Another disease particularly amenable to both prophylaxis treatment by the methods and agents of the present invention is HIV. Trans epithelial infection by HIV is believed to be mediated by dendritic cells, which become infected with the virus and carry the virus to lymph nodes where interactions between dendritic cells and T lymphocytes as described above lead to infection of T lymphocytes. It is known that bone marrow-derived dendritic cells facilitate the creation of a population of CD 4+ T lymphocytes, which are a major reservoir of HIV infection. Methods and agents of the present invention which block the migration of dendritic cells from peripheral sites, where dendritic cell HIV infection occurs, can interrupt the endogenous spread of infection; suppressing further dendritic cell migration inhibits the dendritic cell-mediated fostering of infected T lymphocyte populations.

In one embodiment of the present invention, antibodies or antibody fragments to p-glycoprotein may be administered to inhibit dendritic cell migration. Antibodies may be

made which bind to the extracellular portion of the p-glycoprotein molecule. Non-limiting examples of suitable antibodies include monoclonal antibodies MRK16 (24), 4E3 (23), and UIC2 (22). Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. Other fragments of such

5 antibodies capable of binding to an epitope on the p-glycoprotein molecule are embraced herein. The anti-p-glycoprotein antibodies of the invention may be cross reactive, *e.g.*, they may recognize p-glycoprotein from different species. Polyclonal antibodies have greater likelihood of cross reactivity. Alternatively, an antibody of the invention may be specific for a single form of p-glycoprotein, such as murine p-glycoprotein. Preferably, such an antibody

10 is specific for human p-glycoprotein.

Various procedures known in the art may be used for the production of polyclonal antibodies to p-glycoprotein. For preparation of monoclonal antibodies directed toward p-glycoprotein, or fragment thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the

15 hybridoma technique originally developed by Kohler and Milstein [*Nature* 256:495-497 (1975)], as well as the trioma technique, the human B-cell hybridoma technique [Kozbor et al., *Immunology Today* 4:72 1983]; Cote et al., *Proc. Natl. Acad. Sci. U.S.A.* 80:2026-2030 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole et al., in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)].

20 In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology [PCT/US90/02545]. In fact, according to the invention, techniques developed for the production of "chimeric antibodies" [Morrison et al., *J. Bacteriol.* 159:870 (1984); Neuberger et al., *Nature* 312:604-608 (1984); Takeda et al.,

Nature 314:452-454 (1985)] by splicing the genes from a mouse antibody molecule specific for p-glycoprotein together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. Such human or humanized chimeric antibodies are preferred for use in therapy of human diseases or disorders (described *infra*), since the human or humanized antibodies are much less likely than xenogenic antibodies to induce an immune response, in particular an allergic response, themselves.

According to the invention, techniques described for the production of single chain antibodies [U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778] can be adapted to produce p-glycoprotein-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries [Huse et al., *Science* 246:1275-1281 (1989)] to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for p-glycoprotein.

In a second, and preferred, embodiment of the present invention, administration of agents to inhibit dendritic cell migration are provided which are known to reverse multiple drug resistance. Suitable agents may be selected from compounds that inhibit this drug transporter, including such non-limiting examples as calcium channel blockers such as verapamil, diltiazem, dihydropyridine, nifedipine, bepridil, and nicardipine; other cardiovascular drugs, including amiodarone, dipyridamole, and quinidine; antimalarial compounds such as quinine, quinacrine, and cinchonine; cephalosporin and other antibiotics such as cefoperazone, ceftriaxone, erythromycin, valinomycin, bafilomycin, and gramicidin; cyclosporins such as cyclosporine A and staurosporine; phenothiazines such as

trifluoperazine, chlorpromazine and fluphenazine; hormones such as tamoxifen, N-desmethyldoxifen, 4-hydroxytamoxifen, toremifene, and progesterone, and other compounds such as terfenadine, monensin, yohimbine, reserpine, cremophor, benzquinamide, estramustine, and tacrolimus.

- 5 In a preferred embodiment, more potent inhibitors of multi-drug resistance than those described above are used in the practice of the present invention. Non-limiting examples include the cyclosporine analog PSC 833, MK 571, and the calcium channel blockers dexverapamil and dextiguldipine. PSC 833 is a cyclosporine analog with greater binding specificity for p-glycoprotein (36); it is 10-fold more potent than cyclosporine A and can
- 10 reverse multi-drug resistance in vitro at concentrations of 0.5 to 2.0  $\mu\text{mol/L}$ . MK 571 is a substrate that antagonizes p-glycoprotein and murine multidrug resistance related protein (MRP), and is a particularly potent inhibitor of the latter. MK 571 is known to be an antagonist of the leukotriene  $D_4$  receptor (54), and as will be seen in the Examples below, has utility for the purposes described herein. Other potent inhibitors of p-glycoprotein include
- 15 S9788, rapamycin, MK 571, GF120918, and SR33557.

In a further embodiment, inhibition of p-glycoprotein in dendritic cells may be achieved by targeting gene expression with antisense oligonucleotides. This approach has been demonstrated feasible in vitro using mouse 3T3 fibroblasts (41).

- As described above, another dendritic cell surface protein that may be antagonized to achieve
- 20 the practice of the present invention is tissue factor (abbreviated TF). Tissue factor is a procoagulant protein present on the surface of numerous cell types, including endothelial

cells, which line the vasculature, and monocytes, which circulate in blood. Increased expression of tissue factor by these cell types has been linked to many thrombotic disorders and pathologic states (see below with regard to increased expression). The majority of monocyte membrane-associated tissue factor is not in an enzymatically active form that can initiate clotting; in order to become active, tissue factor must form an active complex with another of the clotting factors, Factor VII or its activated form, Factor VIIa. This complex then leads to clot formation.

Also as described above in the instance of antagonists of p-glycoprotein, tissue factor may also be antagonized in order to inhibit dendritic cell mobility. This may be achieved by the use of antibodies and fragments against tissue factor. A monoclonal antibody against tissue factor that inhibits migration of DC is VIC7. Compounds known to inhibit tissue factor activity include the dilazep (42) and retinoic acid (43). In a further embodiment, inhibition of tissue factor expression in dendritic cells may be achieved by targeting gene expression with antisense oligonucleotides to tissue factor (49). A further strategy on interfering with tissue factor activity in order to suppress dendritic cell migration is through the use of purified, nonlipidated fragments of soluble recombinant human TF. Migration was inhibited in the presence of a soluble, recombinant form of human TF representing the complete amino acid sequence of the extracellular region or fragments thereof, expressed in *E. coli* and used in delipidated form. Soluble TF impeded migration by  $69 \pm 2\%$  in eight independent experiments when used at  $5\text{--}20\text{ }\mu\text{g/ml}$ . Only fragments containing amino acid residues that are carboxyl to residue 202 blocked reverse transmigration effectively. This finding agrees well with the location of the epitope for mAb VIC7, which is between amino acids 181-214.

The finding that both anti-TF mAb and soluble TF inhibit reverse transmigration is consistent with a model in which the binding of endogenous TF on monocytes to an essential ligand is prevented by anti-TF mAb and is competed with by soluble recombinant TF. The competitive binding sites contained in the region spanning 202-219.

- 5 Enhancing the effectiveness of vaccination and other forms of immunotherapy is another feature of the present invention. This aspect is carried out using methods and agents which enhance or activate the dendritic cell membrane proteins p-glycoprotein and tissue factor. Agents useful for this utility include activators of p-glycoprotein, such as bromocriptine (44), N-acetyl-leucine<sub>n</sub>-tyrosine amide where n=1-6 (45), and cytarabine (46) are preferred.
- 10 Suitable activators of tissue factor include endotoxin (47), silver ion (47), and N,N'-dimethyl- $\gamma,\gamma'$ -dipyridylum dichloride (48). Preferably, these agents are admixed with the immunogen to be used for vaccination.

- A further utility of one aspect of the present invention is in the immunotherapeutic treatment of cancer. Numerous studies and trials involve attempts to initiate or enhance an immune
- 15 response in the patient toward the patient's own tumor. However, these regimens have provided little positive results, often because the patient is unable to mount an effective immune response to the tumor antigens. In this case, effective antigen presentation maybe suppressed. The methods and agents of the present invention directed towards the enhancement of the immune response and increasing the effectiveness of vaccines has
- 20 particular utility in the enhancement of therapeutic vaccines for cancer.

In another embodiment of the present invention, methods and agents may be used to increase dendritic cell migration in order to achieve an enhancement of the development of immunity or in a subsequent immune response to an antigen. This would be carried out using agents that increase transport of endogenous substrates for p-glycoprotein that mediate dendritic cell migration. Enhanced development of immunity is particularly useful in enhancing the effectiveness of a vaccine, such as that for viruses such as poliomyelitis, rubella, measles, variola, and varicella, and especially poorly immunogenic immunogens such as influenza; bacteria such as *Borrelia burgdorferi*, the causative pathogen of Lyme disease, and other organisms including parasites such as malaria. Furthermore, the invention herein is also useful in the various newer immunotherapeutic approaches to the prevention and control of other infectious diseases such as HIV and hepatitis, and immunotherapies of various cancers (infra). In these approaches, potentially immunogenic components of the virion or tumor are prepared by various means and introduced into the patient in an attempt to develop immune recognition of the pathologic antigen(s). Often, the patient is immunocompromised and cannot mount an effective immune response to the antigen. One embodiment of the methods and agents of the present invention provide a means for increasing the delivery of antigen to the immune system by increasing or enhancing the migration of dendritic cells which have processed the antigen to the lymphatic vessels, where they may subsequently encounter T lymphocytes and induce cellular and humoral immunity.

The route of delivery of the agents of the present invention may be selected by one skilled in the art to provide the optimal contact with dendritic cells involved in the etiology of the condition to be treated or the route of exposure of the immunogen. As examples, contact dermatitis, psoriasis, and other topical conditions may be treated by application of the agent

of the present invention to the skin. Allergic conjunctivitis may be treated by application of the agent to the eye. Parenteral or other routes of administration which results in generalized delivery of the agent is also provided. Bronchiolar and pulmonary exposure may be achieved by aerosol and fine powder inhalation, respectively.

- 5 In yet another aspect of the present invention, provided herein are pharmaceutical compositions of the above agents. Such pharmaceutical compositions may be for administration for injection, or for oral, pulmonary, nasal or other forms of administration. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of a low molecular weight component or components, or derivative
- 10 products, of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (*e.g.*, Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol)
- 15 and bulking substances (*e.g.*, lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hylauronic acid may also be used. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and derivatives. *See, e.g.*, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack
- 20 Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form.

Contemplated for use herein are oral solid dosage forms, which are described generally in Remington's Pharmaceutical Sciences, 18th Ed.1990 (Mack Publishing Co. Easton PA 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid  
5 encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (e.g., U.S. Patent No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, K. In: *Modern Pharmaceutics* Edited by G.S. Banker and C.T. Rhodes Chapter 10,  
10 1979, herein incorporated by reference. In general, the formulation will include the component or components (or chemically modified forms thereof) and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

In a further embodiment of the present invention, methods and agents are provided for the  
15 augmentation of the migration of monocytes, by enhancing the functioning of the monocyte membrane protein p-glycoprotein. The selection of suitable agents and routes of delivery are as those described above in relation to modulating the same membrane protein on dendritic cells. This aspect of the invention has utility in the treatment of various disorders, preferably chronic inflammatory conditions which are not necessarily, but may be, initiated or  
20 promulgated by antigens and antigen-specific immune cells such as cytotoxic T lymphocytes. For example, the development and progression of atherosclerotic lesions involves the progressive accumulation and retention of monocytes (which become tissue macrophages) and other cell types in lesions; the local inflammation mediated by their secretory products

which attracts more pro-inflammatory cells, together with secretion of growth factors, is a pathophysiologic basis for vessel occlusion.

5 The methods and agents of the present invention directed to increasing monocyte migration has particular utility in preventing the retention of monocytes in inflammatory sites within the body, thus limiting the extent of inflammation. Other examples of diseases and condition in which accumulation and retention of monocytes and resultant production of inflammatory mediators, growth factors and chemoattractants for additional inflammatory cells include rheumatoid arthritis and the granulomatous diseases.

10 It is a further object of the present invention to provide methods for identifying agents and compound useful in modulating the migration of dendritic cells. As a consequence of the discovery by the inventors herein of the presence of p-glycoprotein on the membrane of dendritic cells and the further discovery of the role of p-glycoprotein and tissue factor in dendritic cell migration, various known methods for measuring the effect of compounds on p-glycoprotein activity may be adapted for use on dendritic cells. For example, dendritic cells  
15 may be isolated from skin explants and loaded with a dye known to be exported by functioning p-glycoprotein (28), such as 3,3'-diethyloxacarbocyanine iodide (DiOC<sub>2</sub>). Export of the dye may be measured in the absence and presence of compounds suspected of inhibiting or enhancing the activity of p-glycoprotein by decreasing or increasing, respectively, the pumping of the dye out of the cell. In a further embodiment, agents useful  
20 for the modulation of dendritic cell migration may be identified by first identifying whether a candidate agent is capable of interacting with p-glycoprotein or tissue factor; and subsequently determining the extent of modulation of the agent on the migration of dendritic

cells in vitro or in vivo. The initial screen may be performed on cells which express p-glycoprotein, such as any one of numerous cancer cell lines.

Subsequently, the effect of the compound on the migration of dendritic cells may be performed as described below or that described in U.S. Patent 5,627,025, incorporated herein  
5 by reference. This method employs human skin explants in culture to measure the effect of compounds on dendritic cell emigrating from the skin explant into the culture medium. The methods disclosed herein are not limited to any particular method but are based upon the newly-identified role of p-glycoprotein and tissue factor on dendritic cells in cell migration.

Likewise, assays may be performed to identify compounds which enhance migration of  
10 monocytes out of tissues. As one non-limiting example, migratory behavior of monocytes may be evaluated in an in vitro model of the blood vessel wall consisting of human umbilical vein endothelial cells (HUVEC) grown on type I collagen gels (see Example I, below). Human blood monocytes, which express p-glycoprotein, transmigrate across the confluent endothelium and enter the collagenous substrate. The extent of migration is measured after  
15 exposure of the monocytes to the candidate. Other methods of measuring the migration of monocytes are known to the skilled artisan and are useful in the practice of the present invention.

In a further example of a method for identifying compounds useful for modulating dendritic cell p-glycoprotein activity, the following steps are carried out:

- 20 i) loading isolated dendritic cells with a detectable substrate of the p-glycoprotein transporter activity;

- ii) exposing the loaded cells to an agent suspected of modulating p-glycoprotein activity;
- iii) measuring the rate of transport of the detectable substrate from the dendritic cells; and
- 5 iv) comparing said rate to the rate of p-glycoprotein transporter activity determined of control dendritic cells not exposed to the agent.

An increase or decreased in the transport rate in the presence of the agent indicates the extent of activity of the agent in increasing or decreasing, respectively, p-glycoprotein activity of said dendritic cells. In a further step, compounds that are active in the above assay, either at increasing or decreasing p-glycoprotein activity, can be additionally assays to determine if they are useful for modulating the migration of dendritic cells by determining the extent of modulation of the agent of the migration of dendritic cells in vitro or in vivo. DC migration may be determined by any of several techniques, such as the emigration rate described in aforementioned U.S. Patent 5,627,025. In another method, compounds may be evaluated directly in the emigration assay (7). Activity may be evaluated in vivo by administration of the agent to an animal, followed by, for example, application of an antigen to the skin which would under normal circumstances induce immunity in the animal or an immune response. The extent of the any reduction in the immune response elicited after administration of the agent is an indication of the extent of inhibition of dendritic cell migration. Similarly, compounds to be evaluated for the enhancement of the immune response may be evaluated in vivo by administering the agent parenterally or to the skin, for example, followed by application of an immunogen. Any resulting enhancement of the immune response as compared with control animals indicates positive activity in enhancing the immune response.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

## MATERIALS AND METHODS

- 5     *Transendothelial Migration Assays* Using a method detailed previously (10, 11), freshly isolated peripheral blood mononuclear cells (PBMC), approximately one-fourth of which are monocytes, were added to confluent monolayers of unstimulated HUVEC grown on type I bovine collagen gels with or without addition of mAb. Cultures were incubated for 1.5 hr; during this time, monocytes, but very few lymphocytes, transmigrate into the collagen (10).
- 10    To examine their subsequent basal-to-apical transendothelial migration, monocytes were allowed to accumulate beneath the endothelium in the absence of added mAb. Then monolayers were washed twice in Medium 199 (Life Technologies, Grand Island, NY) to remove nonadherent cells from the apical surface, and individual wells received aliquots of Medium 199 containing 20% heat-inactivated human serum with or without added
- 15    monoclonal antibodies (mAbs) as indicated. After 24 hr of incubation, cultures were washed twice in Medium 199 and control medium or medium containing mAb was replenished. After a total time of 48 hr in culture, samples were fixed for microscopic analysis. Differential interference contrast optics were used to quantitate the number of mononuclear phagocytes (MP) that were beneath the endothelial monolayer (11) in at least five high-power
- 20    fields per sample. Each experiment was conducted with six replicates per parameter tested.

*Skin Cultures* Human split-thickness skin was obtained from the New York Firefighter's Skin Bank (New York Hospital-Cornell Medical Center) from cadavers within 24 hr of death, or

from patients undergoing plastic surgery, and was authorized for use in research. Generally, dermatomes were approximately 300-mm thick, including both epidermis (about 100-mm) and a portion of the dermis. Skin was prepared, cultured, and emigrated cells were quantitated as previously described (7). Each explant was trimmed to 400-mm<sup>2</sup> and floated in 3 to 6 ml culture medium. When used, mAbs or verapamil (Sigma) were added to the culture medium at the onset of culture, and cultures were incubated undisturbed until the indicated day of analysis. Verapamil was prepared as a concentrated stock in methanol. Final methanol concentration in cultures was 0.03% (vol/vol).

*Immunostaining and Flow Cytometry* Skin explants were separated into epidermal and dermal sheets by treatment with 0.5 M ammonium thiocyanate for 20 minutes at 37° C (12). Alternatively, skin samples were snap-frozen in OCT compound (Miles; Elkhart, IN) and then used for the preparation of thick sections (approximately 100  $\mu$ m) cut parallel to the epidermis (6). After either procedure, sheets of skin were fixed in acetone for 5 min. before staining. Sheets of epidermis or thick sections were incubated overnight at 4°C in Eppendorf tubes with CY3-conjugated goat anti-mouse IgG (1:200; Jackson Immunoresearch Laboratories, Inc.), 1:20 FITC-conjugated anti-MHC II (Becton Dickinson), or anti-MHC II 9.3C9 hybridoma supernatant. Tissues were washed three times in PBS containing 1% Tween 20, 15 min. each at room temperature. For detection of 9.3C9 when it was used, CY3-conjugated goat anti-mouse IgG (Jackson Immunoresearch, Inc.) was added for 2 hr at 4° C, followed by three washes as just described. Staining for flow cytometry was carried out as previously detailed (7).

In some experiments, explants were incubated in dispase II (Boehringer Mannheim) for 45 min. at 37°C to separate the epidermis from dermis. Pieces of epidermis were submerged in 0.05% trypsin/0.53 mM EDTA for 45 min. Single cell suspensions were generated by gently pressing epidermal pieces through a fine mesh. Aliquots of digested cells were incubated with FITC-labeled anti-MHC II for 30 min. on ice, washed twice, and analyzed by flow cytometry.

*Immunoblots* Emigrated DC were purified by negative selection: anti-CD3 mAb was incubated with emigrated cells, and T lymphocytes were removed using anti-mouse IgG-conjugated magnetic beads (Dynal). The purity of the resulting population was verified by flow cytometric evaluation after staining with PE-conjugated anti-MHC II (Becton Dickinson). Microsomal membranes from 5 million thus purified DC, 25 million PBMC, or 5 million HUVEC were prepared as described (13), subjected to SDS-PAGE (4-12% gradient gel) under reducing conditions, and electroblotted onto a nitrocellulose membrane. The membrane was probed with 5 mg/ml C219 anti-MDR-1 mAb (Centocor) and visualized using peroxidase-conjugated anti-mouse IgG, followed by ECL substrate (Amersham).

*Efflux Assays* A previously published method to detect functional MDR-1 (14) was employed with minor modifications. Emigrated cells were incubated in RPMI-1640 containing 20 ng/ml DiOC<sub>2</sub> (Molecular Probes) for 15 min. at 37 °C, washed twice in ice cold medium, resuspended in 10% fetal bovine serum/RPMI-1640 containing no additives or with addition of 10 mg/ml UIC2 mAb (Coulter). After 90 min. of incubation at 37 °C, cells were washed and immediately placed on ice for labeling with PE-conjugated anti-MHC II mAb and analysis by flow cytometry.

**EXAMPLE 1:        Role of MDR-1 in basal-to-apical transendothelial migration of mononuclear phagocytes.**

The present inventors were drawn to investigate the role of p-glycoprotein in dendritic cell migration by a set of observations with blood monocytes, now known to be a progenitor of DC when appropriately stimulated with cytokines (15-19). In these initial experiments, the migratory behavior of monocytes was examined in an in vitro model of the blood vessel wall consisting of human umbilical vein endothelial cells (HUVEC) grown on type I collagen gels (10, 11). Human blood monocytes, which express MDR-1 (20, and data not shown), transmigrate across the confluent endothelium and enter the collagenous substrate. This initial migration into the collagen, which is completed within one hr, was unaffected by the addition of anti-MDR-1 mAb, but was partially blocked by mAbs against the  $\beta_2$  chain of integrins (CD18) and CD31, as described (10, 11) (Fig. 1A).

After a brief residence in the collagen, a majority of subendothelial MP retrace the overlying, intact endothelium with a  $t_{1/2}$  of 24 to 48 hr (21). This reverse transendothelial migration was not prevented by mAb to CD18 or CD31 (Fig. 1B). However, screening of mAbs (> 100) submitted to the Vth International Workshop for Human Leukocyte Differentiation Antigens revealed that neutralizing mAbs to MDR-1 strongly inhibited reverse transmigration. UIC2 (22), 4E3 (23), and MRK16 (24) against extracellular domains of MDR-1 inhibited reverse transmigration by 50%, 64%, and 88%, respectively (Fig. 1B). MRK16, used here as F(ab')<sub>2</sub> fragments, was the most potent blocking mAb, exhibiting maximal levels of inhibition using as little as 0.5 to 1.0 mg/ml. C219 (25), which binds an intracellular epitope of MDR-1, had no effect. Preincubation of monocytes with MRK16,

and subsequent removal of unbound mAb before addition of these cells to the endothelium blocked reverse transmigration as well as when the mAb was present continuously during the assay (Pre-MRK16, Fig. 1B). No other mAbs screened inhibited reverse transmigration, except one against tissue factor.

5     **EXAMPLE 2:       Role of MDR-1 in mobilization of DC and T lymphocytes from skin.**

During reverse transmigration, MP migrate across endothelium in a basal-to-apical direction, a movement that is reminiscent of the normal trafficking of DC from the periphery into afferent lymphatics. A panel of mAbs, including those against MDR-1, was applied to  
10     cultured human skin explants. Appearance of DC and T lymphocytes in the culture medium of the explants was reduced by  $71 \pm 9\%$ , and  $79 \pm 16\%$  when the skin was incubated in the presence of MRK16 at concentrations of 2 and 10 mg/ml, respectively (Fig. 2A). Verapamil, a drug that antagonizes MDR-1 transport (26), reduced DC and T lymphocyte accumulation in the culture medium by  $52 \pm 19\%$  at 10 mg/ml (20 mM) (Fig. 2A). The anti-MDR-1 mAb  
15     UIC2 also blocked migration, by  $75 \pm 2\%$  when used at 2 mg/ml (Fig. 2A). An isotype matched control mAb against cadherin 5 (Hec 1) (11) that does not react with DC or T cells had no effect. Moreover, isotype-matched mAbs against other DC surface proteins--CD18 (IB4), CD31 (hec7), and MHC I (W6/32)--also did not inhibit the migration significantly (Fig. 2B). Under all conditions, the cellular content of émigrés ranged from 40 to 60% DC,  
20     with the remaining population being T lymphocytes. Thus, antagonists of MDR-1 blocked migration of DC and T lymphocyte uniformly. Cell viability in all groups was  $>90\%$ , as assessed by trypan blue exclusion.

**EXAMPLE 3: Expression of MDR-1 by DC.**

T lymphocytes are known to express MDR-1 (14, 20, 27), but its expression by DC has not been reported previously. Immunostaining for MDR-1 was observed on DC in the epidermis (Fig. 3A and C). The same cells also stained positively for the DC marker MHC II (Fig. 3B and C). No other cell types in the skin, such as keratinocytes or fibroblasts, showed positive staining for MDR-1 in these experiments. For these studies, skin was cultured in the presence of MRK16 for two days, as for migration analysis. Then staining was carried out on tangential sections prepared from snap-frozen skin, applying only anti-mouse Cy3-conjugated detection antibody. This approach illustrates not only the presence of MDR-1 on epidermal DC in situ, but also demonstrates that mAb applied during culture gains sufficient access to the epidermis. Skin incubated with isotype-matched control mAb hec 1 did not show positive staining.

Flow cytometric analysis of the cells that emigrated from control explants using double labeling with anti-MDR-1 (using either MRK16 or UIC2) and anti-MHC II mAbs demonstrated high levels of MDR-1 on all DC in some experiments (Fig. 4A) or on a majority, but not all, DC in other experiments (Fig. 4B). The fraction of DC staining positively was always greater than the fraction of positively stained subset of T lymphocytes (Fig. 4C).

Expression of authentic MDR-1 by DC was evident in two assays. To document expression of functional MDR-1 (28), DC were loaded with a synthetic, fluorescent substrate for MDR-1, DiOC<sub>2</sub>. At 37 °C, but not 4 °C, DC transported this substrate into the surrounding medium, showing a log decrease in fluorescence intensity (Fig. 4D), and this efflux was

inhibited by anti-MDR-1 mAb UIC2 (Fig. 4D). To identify MDR-1 molecules in DC, crude membranes were prepared and immunoblotted using mAb C219, another mAb that reacts with MDR-1. A broad band of approximately 170 to 180 kDa, the reported molecular weight of MDR-1 was identified (Fig. 4E). The band observed matched that seen in membranes from PBMC and was not observed in HUVEC (Fig. 4E), which do not express MDR-1.

**EXAMPLE 4: Effect of Verapamil on the DiOC<sub>2</sub> Efflux from Dendritic Cells**

Figure 5 further documents that functional p-glycoprotein is expressed by dendritic cells and that p-glycoprotein-mediated transport can be blocked by agents, particularly verapamil and a monoclonal antibody against p-glycoprotein, that are known to inhibit p-glycoprotein transport activity. As described in the Brief Description of Figure 5, dendritic cells were loaded with the p-glycoprotein synthetic substrate 3,3'-diethyloxacarbocyanine iodide (DiOC<sub>2</sub>), and flow cytometry performed as described in Example 3. Approximately 90% of the dye was transported out of the dendritic cells incubated in the absence of p-glycoprotein inhibitors, as indicated by the one-log decrease in fluorescence intensity in the FL1 channel (x-axis) (compare Panel C to D). However, transport of the dye was completely prevented in the presence of verapamil (Panel E) and the anti-p-glycoprotein monoclonal antibody UIC2 (Panel F).

**Example 5: Effect of MDR-1 antagonists on maturation and retention of DC in epidermis.**

To examine the step at which emigration of DC from the explants was inhibited by anti-MDR-1 mAb, epidermal and dermal sheets prepared from cultured skin explants were stained for MHC II (29, 30). Compared to the number present at the onset of culture (Fig.

6A, B), the number of DC remaining in the epidermis after three days of culture under control conditions was decreased by 56% (Fig. 6A, C). In explants cultured in the presence of MRK16 (Fig. 6A, D), UIC2 (Fig. 6A), or verapamil (Fig. 6A), a more limited reduction in DC density of 13%, 11%, and 25%, respectively, were observed. Thus, antagonism of MDR-1 results in retention of DC in the epidermis.

In cultures treated with anti-MDR-1 mAb MRK16, some DC retained in the epidermis appeared morphologically similar to epidermal DC before the onset of culture (compare Fig. 6B and D), but others showed evidence of maturation resembling that observed under control conditions (Fig. 6C and data not shown), including an increase in cell size and greater intensity of staining for MHC II (4, 29, 30). Flow cytometric analysis of DC enzymatically removed from the epidermis after culture in the presence of anti-MDR-1 mAb MRK16 or under control conditions indicated that the maturation of DC, which usually accompanies their mobilization, was not inhibited by anti-MDR-1, as exemplified by the up regulation of MHC II in the majority of DC in the presence or absence of MRK16 (Fig. 7). Thus, antagonism of MDR-1 strongly impedes the migration of DC, but only inconsistently or negligibly suppresses their maturation.

**EXAMPLE 6: Effect of Anti-Tissue Factor Antibodies on Emigration of Dendritic Cells and T Lymphocytes from Skin Explants**

Explants of human skin were floated in culture medium without added monoclonal antibody or in medium containing anti-tissue factor monoclonal antibody VIC7 or control antibodies against cadherin 5 or carcinoembryonic antigen (CEA). After three days of incubation, DC

and T lymphocytes that appeared in the culture medium were collected. Each experiment was conducted with 3-4 replicates per parameter tested. The number of emigrated cells recovered from individual control explants was typically  $5 \times 10^5$ . To compare data from different experiments, the mean number of emigrated cells in the absence of added  
5 monoclonal antibody in each experiment was set equal to 1.0 and relative values were obtained for the remaining data. Cumulative results from three independent experiments are shown in **Figure 8**. These experiments show that monoclonal antibody to tissue factor reduces the emigration of cells from skin explants by about 50%.

**EXAMPLE 7: Effect of P-glycoprotein Antagonists on Dendritic Cell Migration  
10 in Vivo**

To test the effectiveness of p-glycoprotein antagonists on dendritic cell migration in vivo, experiments were carried out in mice. First, the murine molecule analogous to p-glycoprotein in mouse DCs was identified. To do this, mouse skin explants, derived from the dorsal aspect of the ear, were cultured in a manner similar to the human skin explants  
15 (50). Dendritic cells (DCs) that migrated out of the lymphatic vessels of these explants were used in efflux transport assays and in flow cytometry. Efflux of the fluorescent substrate Fluo-3 for p-glycoprotein-related molecules was observed (**Fig. 9A**, left panel), and flow cytometric evaluation using specific antibodies demonstrated the expression of murine multidrug resistance related protein (MRP) (**Fig. 9B**). That MRP was the major p-  
20 glycoprotein-related transporter was further evident from the observation that MRP knock-out mice (51) failed to efflux the fluorescent substrate Fluo-3 (**Fig. 9A**, right panel).

The pharmacologic compound MK 571 is a substrate that antagonizes p-glycoprotein and MRP, but is a particularly potent inhibitor of the latter (52). Thus, MK 571 was added to cultures of human skin explants and mouse ear explants. Migration of DC was blocked by 58% and 62%, respectively (Figs. 10 A,B).

- 5 MRP knock-out mice and age-matched wild-type mice were employed in a contact sensitivity assay to determine whether MRP mediated the migration of murine DCs in vivo. Dendritic cells were analyzed for their transport of the contact sensitizer FITC to lymph nodes 24 h after its application to the skin of mice, using the contact sensitivity assay previously described (53). Dendritic cell accumulation in the lymph nodes of sensitized mice was reduced by 87% in the absence of MRP expression (Fig. 11A).
- 10

- Next, using wild-type mice, MK 571 was administered i.v. (50 mg / kg body weight) four hours prior to application of the contact sensitizer FITC to determine whether pharmacologic inhibitors of MRP and p-glycoprotein are effective in suppressing DC migration. Indeed, administration of MK 571 reduced accumulation of FITC-bearing DCs in the draining lymph node by 64% (Fig. 11B). These data indicate that pharmacologic antagonism of p-glycoprotein suppresses dendritic cell migration.
- 15

This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all respects illustrative and not restrictive, the scope of the

invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

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WHAT IS CLAIMED IS:

- 1 1. A method for decreasing the migration of dendritic cells from the peripheral tissues to  
2 the lymphatic vessels by contacting said dendritic cells with an effective amount of an  
3 agent capable of inhibiting the activity of a dendritic cell membrane protein selected  
4 from the group consisting of p-glycoprotein, tissue factor and the combination  
5 thereof.
- 1 2. The method of claim 2 wherein said agent is an antibody which binds to p-  
2 glycoprotein, an antibody fragment which binds to p-glycoprotein, an agent which  
3 blocks transport activity of p-glycoprotein, an antagonist of p-glycoprotein, or an  
4 antisense oligonucleotide to p-glycoprotein.
- 1 3. The method of claim 3 wherein said antibodies or antibody fragments which bind to  
2 p-glycoprotein are selected from the group consisting of MRK-16, UIC2, 4E3, and  
3 antibody fragments thereof which bind p-glycoprotein.
- 1 4. The method of claim 1 wherein said agent is capable of reversing multidrug  
2 resistance.
- 1 5. The method of claim 1 wherein said agent is a calcium channel blocker, reserpine,  
2 quinine, quinidine, quinacrine, cinchonine, cyclosporine and cyclosporine analogs,  
3 yohimbine, phenothiazines, tamoxifen, N-desmethyldoxifen, 4-hydroxytamoxifen,  
4 toremifene, progesterone, cephalosporin antibiotics, erythromycin, valinomycin,

5           gramicidin, bafilomycin, amiodarone, dipyridamole, quinidine, cortisol, terfenadine,  
6           cremophor, benzquinamide, estramustine, monensin, tacrolimus, GF120918, S9788,  
7           rapamycin, MK 571, or SR33557.

1       6.     The method of claim 5 wherein said calcium channel blocker is selected from the  
2           group consisting of verapamil, diltiazem, azidopine, dihydroperidine, dexverapamil,  
3           nifedipine, bepridil, nicardipine, dextiguldipine, and combinations thereof.

1       7.     The method of claim 5 wherein said cyclosporin is cyclosporine A, staurosporine,  
2           PSC 833, and combinations thereof.

1       8.     The method of claim 5 wherein said phenothiazine is selected from the group  
2           consisting of trifluoperazine, chlorpromazine, fluphenazine, and combinations thereof.

1       9.     The method of claim 1 wherein said agent is an antagonist of tissue factor.

1       10.    The method of claim 9 wherein said agent is an antibody or an antibody fragment to  
2           tissue factor, a tissue factor antisense oligonucleotide, a fragment of tissue factor, or a  
3           recombinant fragment of tissue factor.

1       11.    The method of claim 9 wherein said agent is selected from the group consisting of  
2           diazepam and retinoic acid.

1       12.    A method for suppressing the development of immunity or an immune response in a  
2           mammal by contacting dendritic cells of said mammal with a therapeutically effective

3 amount of an agent capable of inhibiting the activity of a dendritic cell membrane  
4 protein selected from the group consisting of p-glycoprotein, tissue factor and the  
5 combination thereof.

1 13. The method of claim 12 wherein said agent is an antibody which binds to p-  
2 glycoprotein, an antibody fragment which binds to p-glycoprotein, an agent which  
3 blocks transport activity of p-glycoprotein, an antagonist of p-glycoprotein, or an  
4 antisense oligonucleotide to p-glycoprotein.

1 14. The method of claim 13 wherein said antibodies or antibody fragments which bind to  
2 p-glycoprotein are selected from the group consisting of MRK-16, UIC2, 4E3, and  
3 antibody fragments thereof which bind p-glycoprotein.

1 15. The method of claim 12 wherein said agent is capable of reversing multidrug  
2 resistance.

1 16. The method of claim 12 wherein said agent is a calcium channel blocker, reserpine,  
2 quinine, quinidine, quinacrine, cinchonine, cyclosporine and cyclosporine analogs,  
3 yohimbine, phenothiazines, tamoxifen, N-desmethyldoxifen, 4-hydroxytamoxifen,  
4 toremifene, progesterone, cephalosporin antibiotics, erythromycin, valinomycin,  
5 gramicidin, bafilomycin, amiodarone, dipyrindamole, quinidine, cortisol, terfenadine,  
6 cremophor, benzquinamide, estramustine, monensin, tacrolimus, GF120918, S9788,  
7 rapamycin, MK 571, or SR33557.

- 1     17.    The method of claim 16 wherein said calcium channel blocker is selected from the  
2            group consisting of verapamil, diltiazem, azidopine, dihydropyridine, dexverapamil,  
3            nifedipine, bepridil, nicardipine, dextiguldipine, and combinations thereof.
- 1     18.    The method of claim 16 wherein said cyclosporin is cyclosporine A, staurosporine,  
2            PSC 833, and combinations thereof.
- 1     19.    The method of claim 16 wherein said phenothiazine is selected from the group  
2            consisting of trifluoperazine, chlorpromazine, fluphenazine, and combinations thereof.
- 1     20.    The method of claim 12 wherein said agent is an antagonist of tissue factor.
- 1     21.    The method of claim 20 wherein said agent is an antibody or an antibody fragment to  
2            tissue factor, a tissue factor antisense oligonucleotide, a fragment of tissue factor, or a  
3            recombinant fragment of tissue factor.
- 1     22.    The method of claim 20 wherein said agent is selected from the group consisting of  
2            dilazep and retinoic acid.
- 1     23.    The method of claim 12 wherein said suppression of immunity or the immune  
2            response inhibits the immune response to an allergen.

- 1     24.     The method of claim 12 wherein said suppression of immunity or the immune  
2             response is used for the treatment of organ transplant rejection, graft-vs.-host disease,  
3             autoimmune diseases, atopic diseases, or HIV infection.
- 1     25.     The method of claim 24 wherein said autoimmune diseases is rheumatoid arthritis or  
2             psoriasis.
- 1     26.     The method of claim 24 wherein said atopic disease is contact dermatitis, food  
2             allergy, allergic rhinitis, allergic conjunctivitis, asthma, or eczema.
- 1     27.     The method of claim 12 wherein said agent is administered by the topical, parenteral,  
2             ophthalmic, nasal, rectal, bronchiolar or pulmonary route.
- 1     28.     The method of claim 27 wherein said parenteral administration intravenous,  
2             intraarterial, intramuscular, subcutaneous, or intradermal.
- 1     29.     A method for increasing the migration of dendritic cells from the peripheral tissues to  
2             the lymphatic vessels by contacting said dendritic cells with an effective amount of an  
3             agent capable of increasing the activity of a dendritic cell membrane protein selected  
4             from the group consisting of p-glycoprotein, tissue factor, and the combination  
5             thereof.
- 1     30.     The method of claim 29 wherein said agent is a p-glycoprotein agonist or a tissue  
2             factor agonist.

- 1 31. The method of claim 30 wherein said agent is bromocriptine, N-acetyl-leucine,  
2 tyrosine amide where n=1-6, cytarabine, endotoxin, silver ion, or N,N'-dimethyl- $\gamma,\gamma'$ -  
3 dipyridylum dichloride.
- 1 32. The method of claim 29 wherein said agent is co-administered with an immunogen to  
2 enhance the development of immunity to said immunogen.
- 1 33. The method of claim 32 wherein said immunogen is a vaccine.
- 1 34. A method for enhancing the development of immunity or an immune response in a  
2 mammal by contacting dendritic cells of said mammal with a therapeutically effective  
3 amount of an agent capable of increasing the activity of a dendritic cell membrane  
4 protein selected from the group consisting of p-glycoprotein, tissue factor, and the  
5 combination thereof.
- 1 35. The method of claim 34 wherein said agent is a p-glycoprotein agonist or a tissue  
2 factor agonist.
- 1 36. The method of claim 34 wherein said agent is bromocriptine, N-acetyl-leucine,  
2 tyrosine amide where n=1-6, cytarabine, endotoxin, silver ion, or N,N'-dimethyl- $\gamma,\gamma'$ -  
3 dipyridylum dichloride.

1 37. The method of claim 34 wherein said agent is co-administered with an immunogen to  
2 enhance the development of immunity to said immunogen.

1 38. The method of claim 37 wherein said immunogen is a vaccine.

2 39. The method of claim 38 wherein said vaccine comprises a viral, bacterial, or parasite  
3 immunogen.

1 40. The method of claim 37 wherein said immunogen is a tumor antigen.

1 41. A method for the identification of agents useful for the modulation of dendritic cell  
2 migration comprising the steps of:

3 i) identifying an agent capable of interacting with the a dendritic cell  
4 membrane protein selected from the group consisting of p-glycoprotein  
5 and tissue factor; and

6 ii) determining the extent of modulation of said agent of step (i) in the  
7 migration of dendritic cells in vitro or in vivo.

1 42. A method for the identification of agents useful for modulating the development of  
2 immunity or an immune response in accordance with the method of claim 41.

1 43. A method for identifying compounds useful for modulating dendritic cell p-  
2 glycoprotein activity comprising the steps of:

- 3 i) loading isolated dendritic cells with a detectable substrate of the p-  
4 glycoprotein transporter activity;  
5 ii) exposing said loaded cells to an agent suspected of modulating p-  
6 glycoprotein activity;  
7 iii) measuring the rate of transport of said detectable substrate from said  
8 dendritic cells; and  
9 iv) comparing said rate to the rate of p-glycoprotein transporter activity  
10 determined of control dendritic cells not exposed to said agent,  
11 wherein an increase or decreased in said rate in the presence of the agent indicates the  
12 extent of activity of said agent in increasing or decreasing, respectively, p-  
13 glycoprotein activity of said dendritic cells.

1 44. A method for identifying compounds useful for modulating the migration of dendritic  
2 cells by carrying out the method of claim 43, followed by determining the extent of  
3 modulation of said agent of the migration of dendritic cells in vitro or in vivo.

1 45. A method for augmenting the migration of monocytes and monocyte-derived cells  
2 from tissues by contacting said monocytes with an effective amount of an agent  
3 capable of augmenting monocyte p-glycoprotein activity.

1 46. A method of treating a chronic inflammatory disease or condition in a mammal by  
2 enhancing the migration of monocytes and monocyte-derived cells from the affected  
3 site of said disease or condition by administration of a therapeutically effective  
4 amount of an agent capable of augmenting monocyte p-glycoprotein activity.

- 1      47.    The method of claim 46 wherein said inflammatory disease or condition is  
2      atherosclerosis, rheumatoid arthritis, or granulomatous diseases.

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FIG. 1A

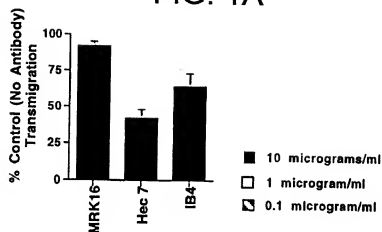


FIG. 1B

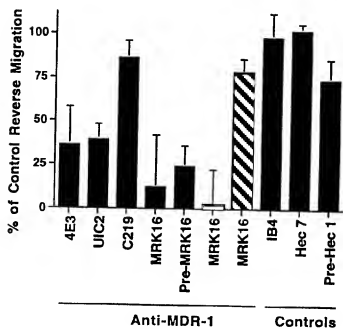


FIG. 2

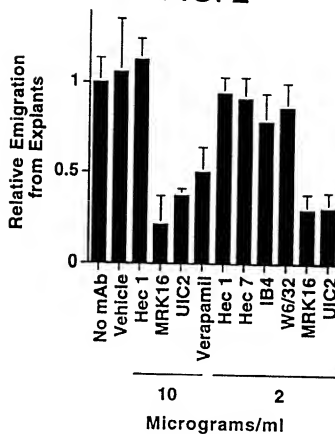


FIG. 3A

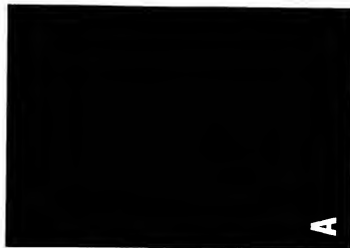
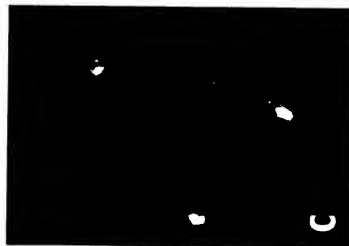


FIG. 3B



FIG. 3C



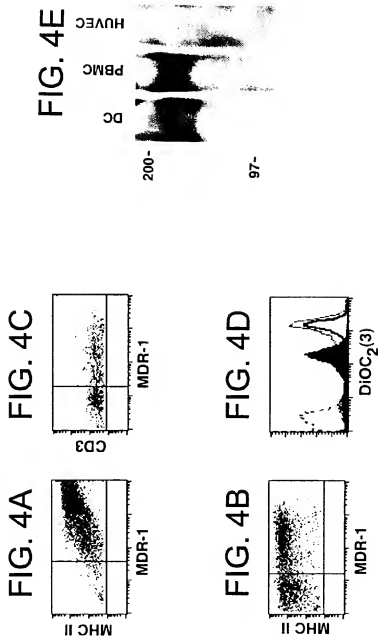


FIG. 5A

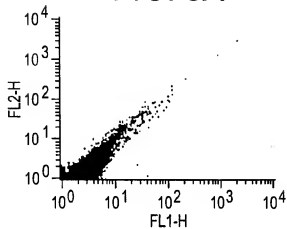


FIG. 5B

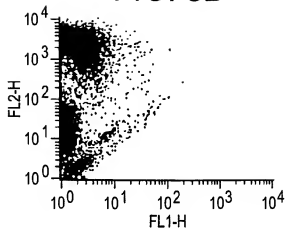


FIG. 5C

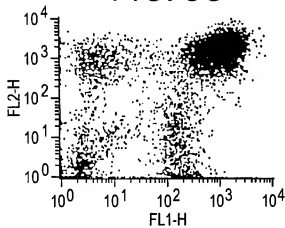


FIG. 5D

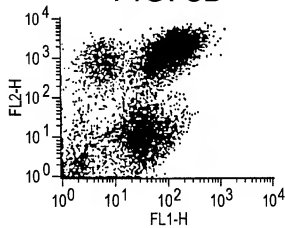


FIG. 5E

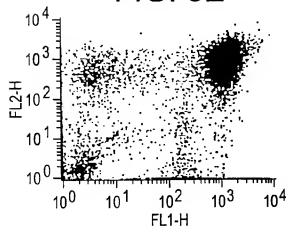


FIG. 5F

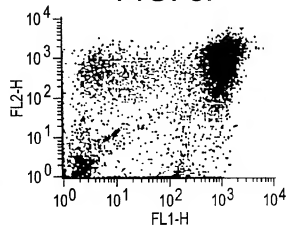


FIG. 6A

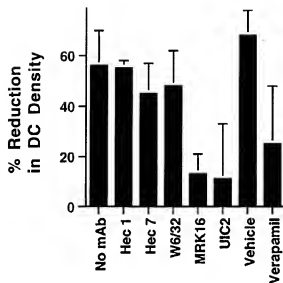


FIG. 6B



FIG. 6C

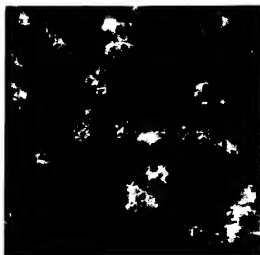


FIG. 6D



FIG. 7A

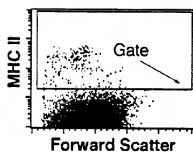
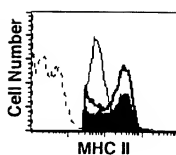
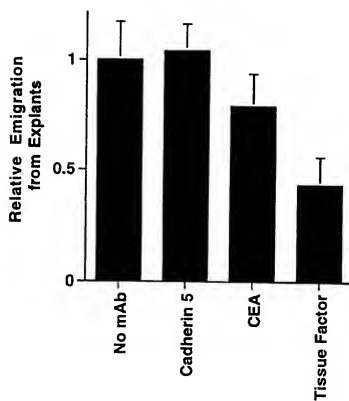


FIG. 7B



- Day 0 (Gated)
- Day 3, MRK16 (Gated)
- Day 3, No mAb (Gated)
- Non-gated Keratinocytes

FIG. 8



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FIG. 9A

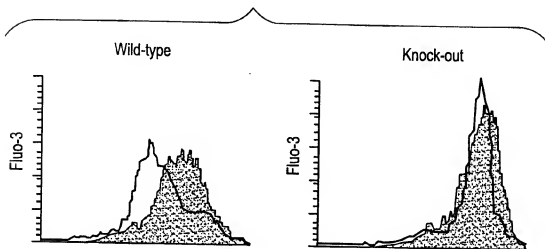
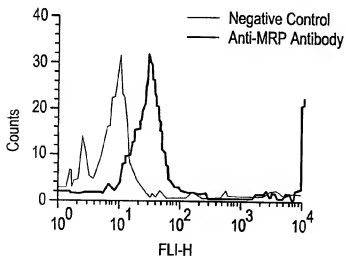


FIG. 9B



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FIG. 10A

HUMAN

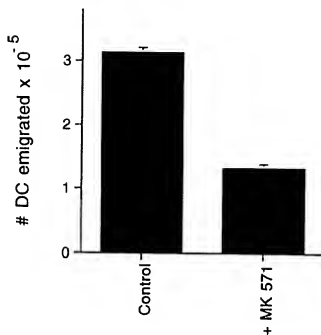
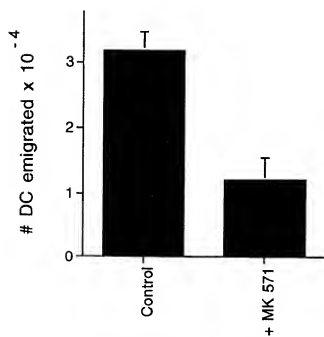


FIG. 10B

MOUSE



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FIG. 11A

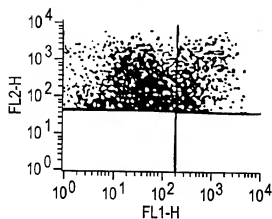


FIG. 11B

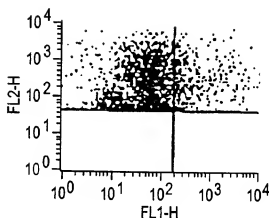


FIG. 11D

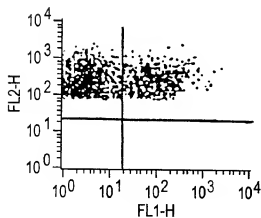
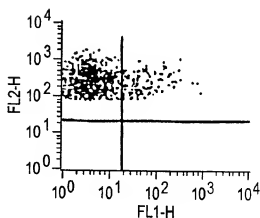


FIG. 11E



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FIG. 11C

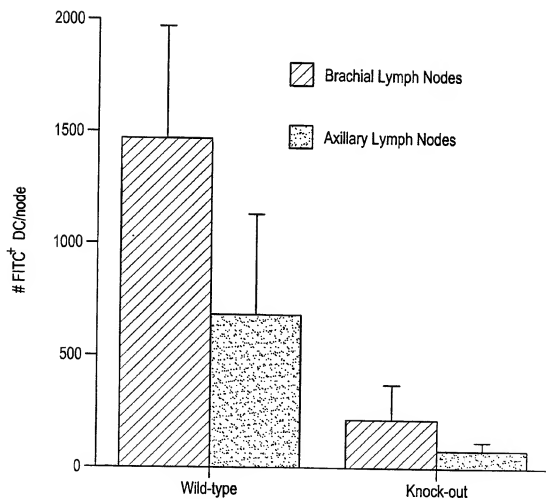
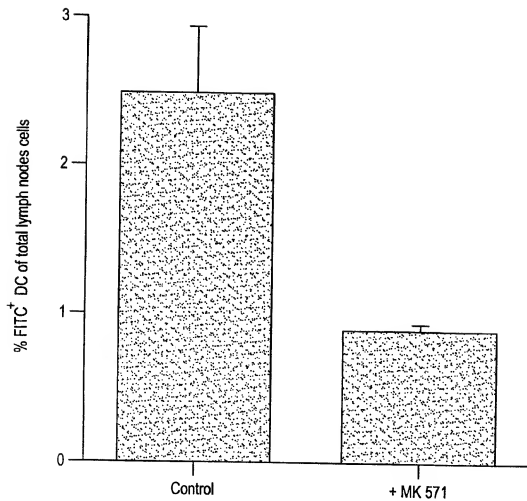


FIG. 11F



## INTERNATIONAL SEARCH REPORT

# INTERNATIONAL SEARCH REPORT

Inventor:   
 PCT/US 99/12681

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6	A61K38/00	A61K39/395	C12N15/11	G01N33/53	A61K39/39
	A61K38/13	A61K38/36	A61K31/20	A61K31/275	C07D279/00
	A61K31/55	A61K39/00	A61K39/002	A61K39/12	A61K39/02

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>RANDOLPH G J ET AL: "Role of tissue factor in adhesion of mononuclear phagocytes to and trafficking through endothelium in vitro." BLOOD, (1998 DEC 1) 92 (11) 4167-77. , XP002112753 abstract page 4167, left-hand column, paragraph 3 - right-hand column, paragraph 1 page 4169, right-hand column, paragraph 2 - page 4170, right-hand column, paragraph 3 page 4172, right-hand column, paragraph 2 - left-hand column, paragraph 4 page 4175, left-hand column, paragraph 1 - right-hand column, paragraph 1 page 4176, left-hand column, paragraph 1 --- -/-</p>	1,9,10, 12,20, 21,23

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex

### \* Special categories of cited documents:

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

20 August 1999

Date of mailing of the international search report

13/09/1999

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Covone, M

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/12681

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 //C07K14/705,C07K14/745,C07K16/36,C07K16/28

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 12633 A (IMMUNEX CORP) 10 April 1997 (1997-04-10) page 2, line 31 - page 3, line 8 page 9, line 29-34 claims 16-19 ---	29-40, 44,45
A	CUMBERBATCH M ET AL: "Tumour necrosis factor-alpha is required for accumulation of dendritic cells in draining lymph nodes and for optimal contact sensitization." IMMUNOLOGY, (1995 JAN) 84 (1) 31-5., XP002112754 page 31, left-hand column, paragraph 1 - page 32, left-hand column, paragraph 1 page 32, left-hand column, paragraph 5 page 34, left-hand column, paragraph 1 - right-hand column, paragraph 2 --- -/-	1-27, 41-44, 46,47

☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

20 August 1999

Date of mailing of the international search report

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Fax (+31-70) 340-3016

Authorized officer:

Covone, M

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/12681

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 01645 A (PEPTECH UK LTD ;LEDGER PHILIP (GB)) 25 January 1996 (1996-01-25)  page 1, line 6-22 page 22, line 23 - page 23, line 22 example 4	1-27, 41-44, 46,47
A	MA J ET AL: "In vivo treatment with anti-ICAM-1 and anti-LFA-1 antibodies inhibits contact sensitization-induced migration of epidermal Langerhans cells to regional lymph nodes." CELLULAR IMMUNOLOGY, (1994 OCT 15) 158 (2) 389-99. , XP002112755 abstract page 392, paragraph 6 - page 394, paragraph 1 page 398, paragraph 3	1-47
A	DATABASE MEDLINE 'Online! US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US LARSEN C P ET AL: "Migration and maturation of Langerhans cells in skin transplants and explants." retrieved from STN Database accession no. 91037811 XP002112758 abstract & JOURNAL OF EXPERIMENTAL MEDICINE, (1990 NOV 1) 172 (5) 1483-93. ,	1-47
A	LAVIE Y. ET AL.: "Agents that reverse multidrug resistance, Tamoxifen, Verapamil, and Cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells" J. BIOL.CHEM., vol. 272, no. 3, 17 January 1997 (1997-01-17), pages 1682-87, XP002112756 abstract page 1686, paragraph 1 page 1687, paragraphs 2,3	1,4-8, 12,15-19
P,X	RANDOLPH G J ET AL: "A physiologic function for p - glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 JUN 9) 95 (12) 6924-9. , XP002112757 the whole document	1-47

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 12681

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
1-40 and 45-47 (completely), 41-44 (partially)  
  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 41-44 (partially) are directed to a diagnostic method practised on the human/animal body and claims 1-40, 45-47 (completely) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 1-40 45-47 (completely), 41-44 (partially)

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal body

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/12681

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9712633 A	10-04-1997	AU 697539 B	08-10-1998
		AU 7392296 A	28-04-1997
		CA 2232865 A	10-04-1997
		CZ 9800977 A	17-02-1999
		EP 0871487 A	21-10-1998
		LV 12085 A	20-07-1998
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		NO 981374 A	03-06-1998
		PL 325964 A	17-08-1998
WO 9601645 A	25-01-1996	AU 2893595 A	09-02-1996
		BG 101126 A	30-09-1997
		CA 2194678 A	25-01-1996
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		EP 0768888 A	23-04-1997
		HU 77290 A	30-03-1998
		JP 10505580 T	02-06-1998
		SK 2697 A	06-08-1997